PHARMACOGNOSTIC AND BIOLOGICAL STUDIES OF THE LEAVES AND STEM-BARK OF *HYMENOCARDIA ACIDA*, TUL. (EUPHORBIACEAE)

BY

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THESIS SUBMITTED TO THE POSTGRADUATE SCHOOL, AHMADU BELLO UNIVERSITY, ZARIA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD 0F THE DEGREE OF MASTER OF SCIENCE IN PHARMACOGNOSY

**DEPARTMENT OF PHARMACOGNOSY AND DRUG DEVELOPMENT,
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AHMADU BELLO UNIVERSITY,
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2004

DECLARATION

I hereby certify that the work reported in this thesis was carried out by me in the Department of Pharmacognosy and Drug Development, A.B.U.Zaria, Nigeria. The works of other investigators are acknowledge and referred to accordingly. I solemnly declare that no part of this thesis has been accepted in substance or concurrently submitted in candidature for nay other degree.

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CERTIFICATION

This thesis entitled PHARMACOGNOSTIC AND BIOLOGICAL STUDIES OF THE LEAVES AND STEM-BARK OF *HYMENOCARDIA ACIDA* TUL.(EUPHORBIACEAE) by Danladi, Halima Balaraba meets the regulations governing the award of the Degree of Master of Science in Pharmacognosy of Ahmadu Bello University, Zaria, Nigeria and is approved for its contribution to knowledge for both literary and research activities.

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DEDICATION

TO ALLAH

The Almighty

AND

TO MY

HUSBAND AND CHILDREN

ACKNOWLEDGEMENT

The author wish to express her sincere gratitude to Dr. E.M. Abdurrahman of the Department of Pharmacognosy and Drug Development, Ahmadu Bello University, Zaria, for his effective supervision of this work, reading of the manuscript, corrections and other advices given to me inspite of his heavy responsibilities.

She wishes to express her sincere gratitude to Dr. Hajara Ibrahim, Department of Pharmacognosy and Drug Development,Ahmadu BelloUniversity, Zaria, for her supervision and assistance of the experimental work and valuable comments.

She also wishes to extend her gratitude to Dr. N. D. Ibrahim , Department of Veterinary Pathology and Microbiology, ABU, Zaria.

The author is also grateful to all the academic staff of the department ofPharmacognosy , Faculty of Pharmaceutical Sciences, A.B.U, Zaria for their interest and encouragement during the course of this work.

Thanks are also due to the technical staff of the Department of Pharmacognosy (most especially Mal. Nuhu), for their cooperation and assistance and to Mr. Benjamin Bello of Shubal Business Centre, Kaduna, for word processing the draft.

In addition, thanks also goes to staff of Herbarium, Department of Biological Sciences, A.B.U. Zaria, for identifying the plant species, also thanks to Mr. Francis, Histopathology Laboratory, Faculty of Veterinary Medicine.

Finally, my deepest gratitude and thanks are extended to my family for their understanding, love, support and encouragement and my friends, other people whose names because of space cannot be mentioned, may Almighty Allah reward them abundantly.

ABSTRACT

This study deals with the pharmacognostic and biological aspects of the leaves and stem bark of *Hymenocardia acida* Tul (Family Euphorbiaceae). Euphorbiceae is a large and heterogeneous family found mainly in the tropics and sub-tropics. H. acida is a savannah shrub or tree with twisted trunk usually less than 4.5 m high but sometimes attaining as much as 9m. it is commonly found in Senegal, Sierra Leone, Togo and Nigeria; widely spread in tropical Africa.

In northern Nigeria and in Zaire, the leaves and stem-bark are used in the treatment of eye infection, chest pains, anaemia, diabetes and purulent sores. Macroscopical, microscopical and phytochemical studies were carried out on the leaves and stem-bark of the plant. Characteristic pharmacognistic features of the plant were found to be polygonal sclereids , frequent non-articulate laticifers and on the average brown (outer surface) of the bark. The sessile covering trichomes, orange or yellow coloured, could served as diagnostic features on the epidemis of the leaves. Other macroscopical and microscopical features could serve in the identification of the plant and provide monograph data for the plant.

The various phytochemical tests revealed the presence of carbohydrates, tannins, alkaloids, flavonoids, terpenes and sterols, saponins and cardiac glycosides in both the stem-bark and leaves. Anthraquinones were present in the leaves but not in the stem-bark. Cyanogenic glycosides, resin and balsams were found to be absent. Evaluation of the crude drug was carried out. The moisture content for the leaves and stem-bark were 20.5%w/w, 8.0%w/w; ash value

4.8%w/w, 11.5; acid insoluble ash value 1.5 w/w, 7.5w/w; water soluble extractive value 42.0%w/w, 44.0%w/w; alcohol soluble extractive value 34.0 %w/w, 37.0 %w/w respectively. These established quantitative values can be used in the preparation of a monograph on the plant and provide indices for identification:

The plant was found to be safe orally, there was no observable LD_{50} which is a dose effective for producing a certain sign in 50% of the animals of a group. The mice, survived the doses greater than 5000mg/kg up to 11000mg/kg. The Histopathological studies from the chronic administration of the plant leaves and stem-bark in rats showed a stimulating effect on haemopoiesis and lympocytes proliferation which could help in ameliorating anaemic conditions. It may also play a role in activating the immune system in the cause of prolong treatment. The result had also shown that the prolonged treatment with the leaves or stem-bark of *H. acida* can induce hepatoxicity.

The results of analgesic activity studies showed a significant and doses dependent analgesic activity when compared to untreated controls ($P< 0.05$), this gave justification for the use of the plant in ethnomedicine for the treatment of pain.

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CHAPTER ONE

1.0 INTRODUCTION

The plant *Hymenocardia acida* belongs to the subclass Dicotyledonae- Crassinucelli, Superorder Malviflorae-Urticales, Subclass Rosidae- Euphorbiales, family Euphorbiaceae (Hutchintson and Dalziel, 1958), recently *Hymenocardia* is classified in the family *Hymenocardiaceae* having only 1 genus (*Hymenocardia*) with 5 species (Watson and Dallwitz, 1992).

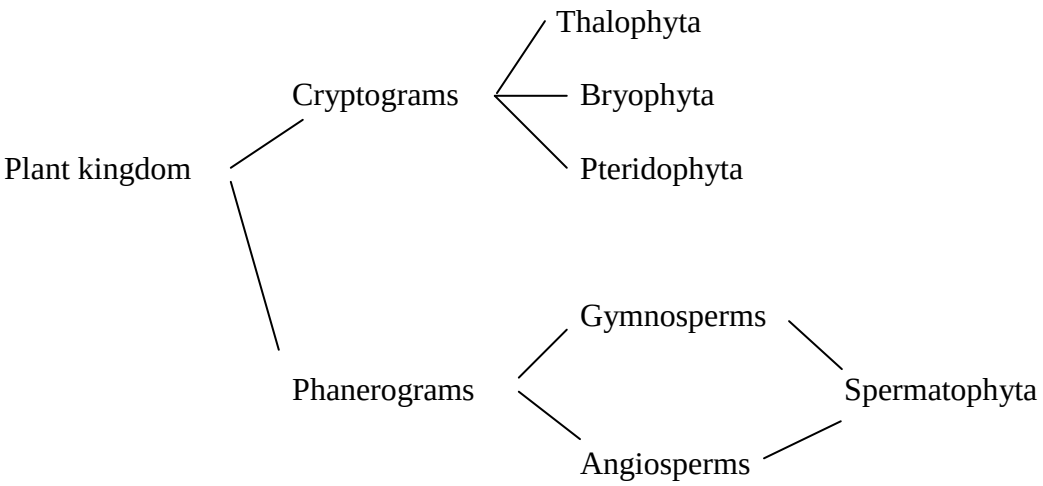
1.1 Description of the Family Euphorbiaceae

Euphorbiaceae is a large and heterogenous family found mainly in the tropics and subtropics. The word Euphorbiaceae is derived from a Greek word that means "membraneous heart" referring to the heart shaped fruits characteristic to all the members of the family. (Eggelling and Dale, 1940).

The family is mainly cosmopolitan found in all parts of the world except in Alpine and Arctic regions. The family is well represented in Africa., Brazil and Indo-Malaysia. Their major habitat is terrestrial, commonly found in the lowland near the riverbank (Irvine, 1961). In England and America the Euphorbiaceae are popularly known as the 'spurge family' (plants with an acid milky juice) (Allain and Allen, 1941).

They are dicotyledenous and belong to the phylum Angiosperms in the vegetative kingdom. The plant kingdom is divided into cryptograms in which there are no live flowers producing seeds, and Phenograms in which their plants develop flowers or flower like structures where seeds are produced. The phenograms include all higher plants and

by this is meant all present day groups which exhibit the highest development and which are usually referred to as the Spermatophyta. This group is again sub-divided into two phyla, the Gymnosperms and the Angiosperms. Figure 1 shows phenograms of higher plants



The Angiosperms can be described further as follows:

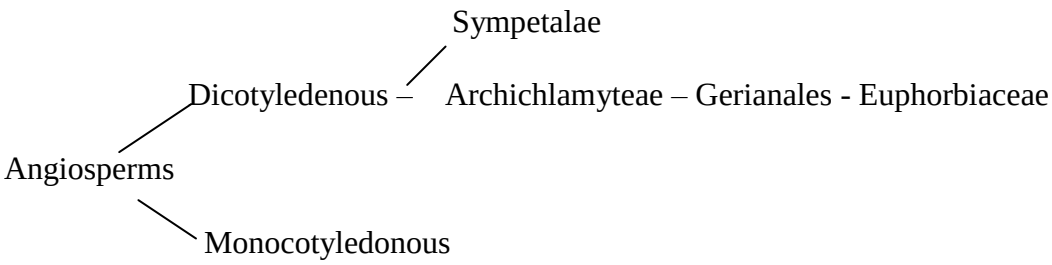


Figure 1: Division and phenogram of plant kingdom (Evans, 1996)

The Euphorbiaceae is the fourth largest of Angiosperm families and is spread over 283 genera and 7,300 species .The Euphobiaceae has been divided into a number of

separate genera, and there still is no unanimity among botanists, but later there is a general agreement that the principal genus of the tribe is *Euphorbia* L. (Alain and Allen, 1941).

The plants are also cheracterised by cyathium.

1.1.1 Cyathium of a Euphorbia

Cyathium is derived form the Greek word, cyathos, meaning a cup, in reference to the cup-like shape which the involucre assumes in many of the species. Early botanists, including Linnaeus, believed the cyathium to be a single perfect flower, with a pistil at the center and five surrounding groups of stamens, the whole enclosed in an envelope of inter-grown petals. Today, as already explained, it is known that the lone pistil is an independent female flower, jointed at its base to a pedicel of varying length, or sometimes sessile, and that each of the staminal clusters is a group of male flowers, every one of them jointed to its separate pedicel. In some *Euphorbias* the individual flowers, male and female alike, have a rudimentary or even a somewhat developed calyx at the base, but this is exceptional and it is more commonly met with other genera of the tribe, notably in Anthostema A. Juss from Senegambia (Allain and Allen, 1941)..

The involucre, which surrounds the entire colony of flowers in each Cyathium, corresponds to a modified whorl of floral leaves or bracts, such as might be found around the base of any flower cluster. And in turn the involucres is often subtended at its base by two or more real bracts, and in most instances the whole cyathium is raised on a peduncle of its own. The whole structure of the cyathium gives the impression that the members of a conventional flower spray had agreed to submerge their individuality telescoping the entire spray into a small unit, discarding all unnecessary parts, gathering around

themselves some of the available leaves or bracts below, fusing these into an enveloping cup or involucre (Fig. 2). It is interesting to know that this theory of the origin of the cyathium was outlined by a German botanist.

Normally the upper part of the involucre is divided into a series of lobes alternating with another series of glands or nectaries, and it is the colour of these and more especially the colour of the glands, which gives the tone to the whole cyathium, a tone particularly vivid and attractive when the glands are a chrome yellow or the like. Yellowish and yellowish-green colours predominate throughout the genus, but involucres of many other shades, such as brown or red or old rose, white or dark purple, also occur. From the upper surface of the glands a little honey is secreted to attract insects, and the smell of this nectar is perhaps even more of an attracting force to the insects than is the colour of the glands.

The lobes in an involucre may vary in number from 4 to 8, and the glands from 2 to 8, or sometimes the whole series is fused into one single gland. The normal count is five lobes and five glands. Some succulent species have only

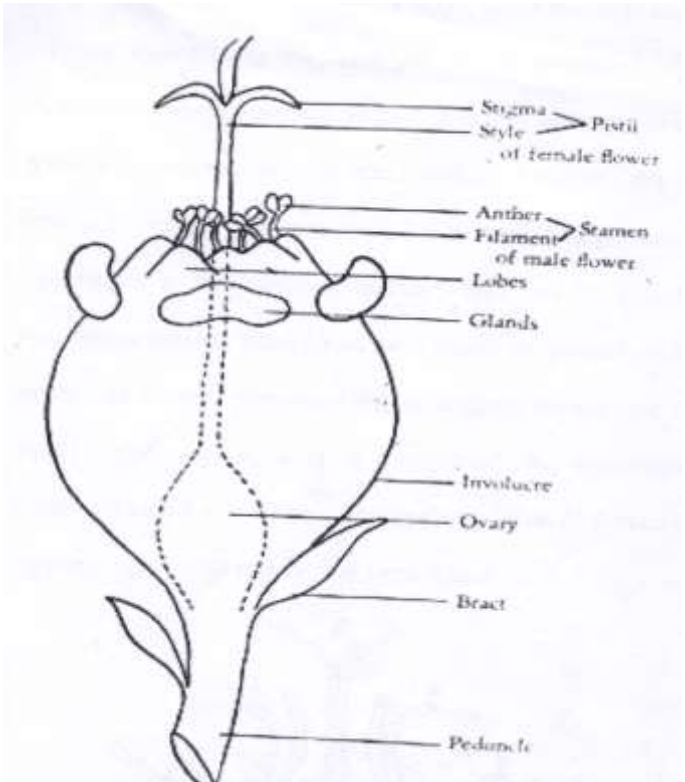


Fig 2 Cyathium of euphorbia (Allain and Allen, 1941)

Four glands, combined with the standard five lobes, and this missing fifth gland is a common feature in many herbaceous species. A very interest combination is found in *E. ornithopus* Jacq and some other species, where five glands are found in some of the cyathia which contain male flowers only, while only four glands occur in the more frequent bisexual cyathia (Allain and Allen, 1941). In the latter the female flower is gradually exerted from involucre on a long recurved pedicel, and the pedicel chooses the precise spot left open by the absence of the fifth gland at which to make its bow to the outside world (Fig. 3).

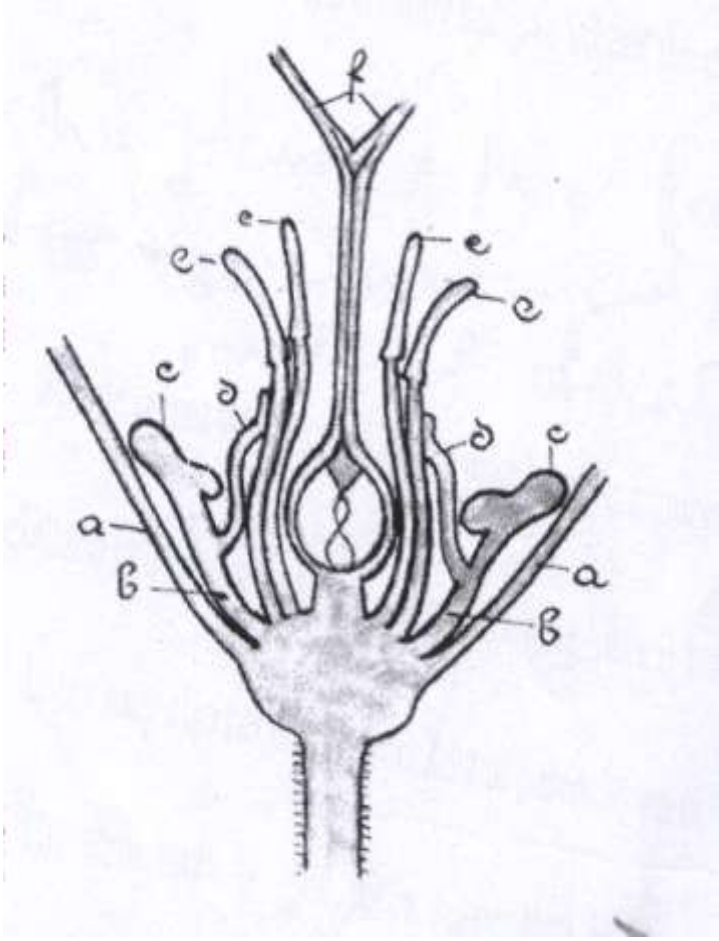


Fig 3 Cross section of Bisexual Cyathium(Allain and Allen,1941)

The Euphobiaceae is characterized by the presence of several types of cell constituents which are of importance in the identification of the plants. The cell constituent of diagnostic importance in the Euphobiaceae is latex. (Allain and Allen, 1941)

1.1.2 Importance and function of latex in the family Euphobiaceae

Latex refers to the suspension (or in certain cases emulsion) of many small particles in a liquid (the refractive index of the suspension or emulsion is different from the liquid in which the small particles are suspended in (Fahn, 1979; Evans, 1996). In the plant kingdom latex occurs in more than 12,500 species belonging to 900 genera and about 20 families. Latex is characteristic of the Dicotyledonae however it occurs in a few families of the Monocotyledonae and in the Pteridophyte genus Regnellidum (Marsilliaceae). Dicotyledons plants latex is characteristically found in the Apocynaceae, Asclepiadaceae, Compositae, Euphorbiaceae, Papaveraceae, Sapotaceae and in a few other families. The monocotyledons containing latex are in the family Araceae, Liliaceae and Musaceae. (Fahn, 1979). The presence of latex is important in the family Euphobiales and serves as an important diagnostic tool for these plants.

The various component of latex include the following:-

Poly isoprene hydrocarbons

Triterpenols and sterols

Fatty acids and aromatic acids (esterified)

Carotenes

Phospholipids

Proteins

Inorganic

Poly isoprene hydrocarbons $(C_5H_8)n$ have 2 isomers occurring in nature in which the methyl groups are cis (rubber) or trans (gutta percha) to the double bond. Also the polyisoprene of rubber contains 500 – 5000 residues while gutta – percha contains only about 100. (Fahn, 1979).

Some substances are found in the latex of specific Euphobiales, these are sugars in the (compositae), starch grains (in *Euphorbia* species), tannins (in *Musa* species), alkaloids (in *Papaver somniferum*), and protein (in *Taraxacum bicorne*); (Fahn, 1979). A large amount of protein occurs in *Ficus callosa* while in *Allium cepa* crystals which show predominantly lipoidal properties occurs, and in *Carica papaya* a proteolytic enzyme, papain is present. The latex of some *Euphorbia* species was found to be rich in Vitamin B, contain abundant oxalate and malate crystals.

The colour of latex is also an important identification parameter and varies in the different plant species. Latex may be white and milky (in *Euphorbia*), brown (in *Cannabis*), orange (in *Chelidonium*), or colourless (in *Morus*, *Nerium*).

Latex can occur in specialised cells called laticifers or cells other than laticifers.

1.1.3 Importance and function of laticifers in the family Euphobiaceae

Laticifers are specialized latex-containing cells (Fig 4) ; their presence is an important tool in the taxonomy of Euphorbiaceae . There are two main types, non-articulated and articulated. This classification has no relationship to taxonomic groups and thus different types of laticifers may be found in different species of one family. The non articulated laticifers are multinucleate. They develop from a single cell which greatly elongates with the growth of the plant. Such laticifers are also termed laticiferous cells. In some plants they develop into more or less straight tubes which are term non-articulated unbranched laticifers. In others they branch repeatedly forming immense systems. These laticifers are termed non-articulated branched laticifers (Fahn, 1979).

Articulated laticifers, also termed laticiferous vessels, consist of simple or branched series of cells, which are usually elongated. The end walls of such cells remain entire or become porous or disappear completely. In the latter case the final result is a large multinucleate structure as in the non-articulated laticifers. The simple articulated laticifers are termed non-anastomosing (unbranched) laticifers, the branched articulated laticifers which form lateral anastomoses with neighbouring ones are termed articulated anastomosing (branched) laticifers. The following are examples of the various types:-

- Non-articulated unbranched laticifers: *Cannabis* (Cannabiuceae); *Urtica* (Urticaceae), *Vinca* (Apocynaceae).
- Non-articulated branched laticifers: *Asclepias*, *Ceropegia*, *Cryptostegia (Asclepiadaceae); Bronssonetia*, *Ficus*, *Maclure* (Moraceae); *Nerium* (Apocynaceae); *Euphobia* (Euphorbiaceae) (Fig. 4).

- Articualted non-anastomosing laticifers, Achras (Sapotaceae); Allium (Liliaceae); Chelidonium (Papaveraceae); Convolvulus, Ipomoea, Dichondia (Convolvulaceae); Musa (Musaceae).
- Articulated anastomosing laticifers: *Argemone*, *Papaver* (papaveraceae); *Carica* (Caricaceae); *Manihot*, (Euphorbiaceae); all or most genera of the Campanulaceae and Lobeliaceae. (Fahn, 1979). Latex occurs sometimes in cells other than laticifers. In the plant *Parthenium argentatum* (Guayula) of the Compositae it occurs in parenchymatous cells which are not morphologically differentiated laticifers (Fahn, 1979).

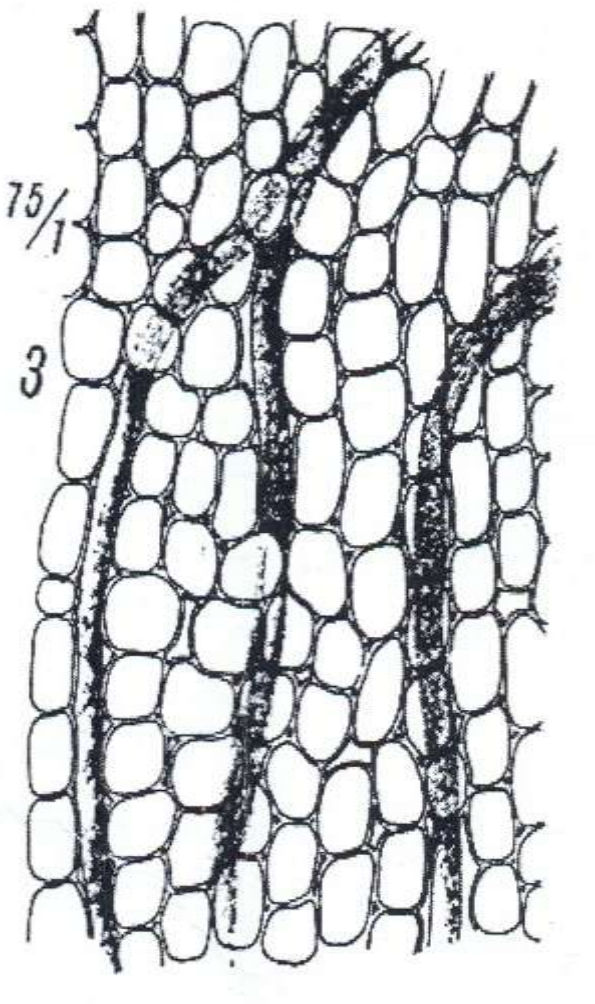


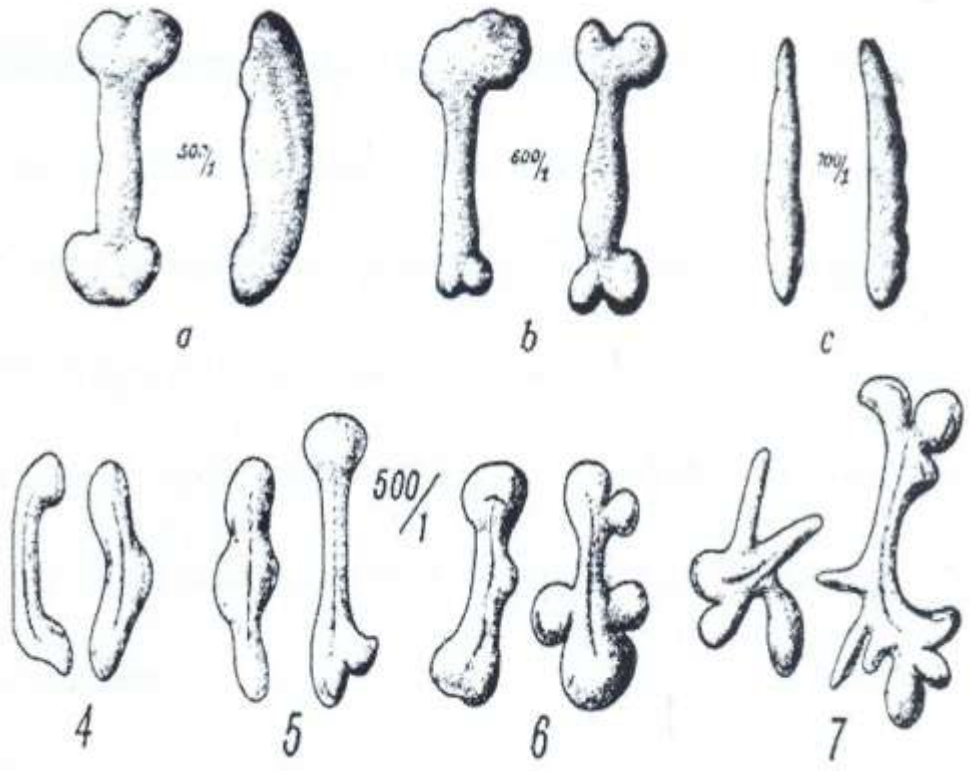
Fig. 4: Latex tubes of a *Euphorbias (* Allain and Allen, 1941).

The functions of laticifers in the plant is primarily secretory, but they apparently produce and store substances which do not participate in the plant metabolism. They also regulate the water balance in the plant and in the transport of oxygen. They most certainly offer protection to the plant in wound healing, defense against herbivores and microorganism. (Fahn, 1979). The biological action of latex are numerous and this depends on the type and plant containing the latex, for example *Calotropis procera* (Asclepiadacea) causes injury to human ocular tissues which may be as a result of specific activity on lysosomal system in the eyes (Fahn, 1979).

1.1.4 Starch grains in laticifers

Starch grains occur in the laticifers of some plants. The starch grains of the non-articulated Euphobiaceae laticifer (Figure 5) possess a specific morphology. The peculiar and variable shapes of the starch grains in laticifers of Euphorbiaceae have been as a result of the evolutionary conditions. The non-articulated laticifer present in only a few families has been interpreted as being of recent phylogenetic origin. It was suggested that elongated laticifer starch grains were derived phylogenetically from the round or oblong grain present in the parenchymatous cells. It was further suggested that the specialization of the grains occurred by the deposition of additional starch at their tips resulting in osteoid grains . Further specialization led to the appearance of multiple lobes at the end of the grains and to development of lobes along the mid-region of the grain. The discoid grains are regarded as the most complex forms.

The fact that starch grains in laticifers differ from those of the other plant cells demonstrates that the laticifers are physically and cytologically independent, although they differentiate from the same meristem (Fahn, 1979).



a. E.caput-medusae L; b. E. burmanni, E. mey; c. E. mauritanica L.; 4. E. drageana. E. mey; 5. E.bamata sweet 6. E. triangularis; 7. Synadenium cupulare L., C. wheeler.

Figure 5: Starch grains from latex of *Euphorbias* (Reproduced Allain and Allen, 1941)

1.1.5 Importance and function of rubber in the family Euphobiaceae

Plants which produce rubber are numerous, however, there may be differences within the same genus e.g. *Euphorbia*, where the tropical species produce and accumulate considerable amount of rubber, while the temperate species of the same genus may produce little or no rubber.

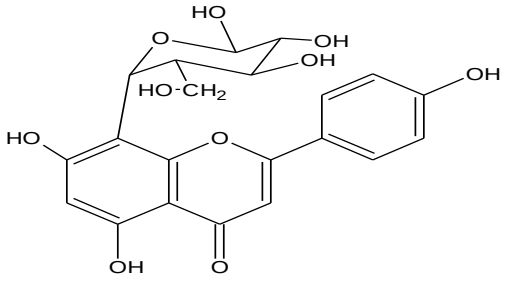
The most important rubber producing plant is para-rubber *Hevea brasiliensis* (Euphorbiaceae). Some other *Hevea* species e.g. *H. benthamia* and *H. guianensis*, also contribute to modern rubber cultivars. Of lesser importance are: Panama rubber – *Castilla ulei* and *C. elastica* (Moracaee); Manicoba – *Manihot glaziovii; M. Heptaphyla M. piauhyensis* and *M. dichotoma* (Euphorbiaceae); Mangabeira – *Hancornia speciosa* (Apocynaceae)' Landolphea – *Landolphia kikii, L. heudelotii* and *L. owariensis* (Apocynaceae); Palay Rubber – *Cryptostegia grandiflora* and *C. madagascariencis* (Asclepiadaceae); Russian Dendelion – *Taraxacum koksaghyz,* Guayule – *Parthenium argentatum* (Compositae).

The presence of latex in a plant does not guarantee the presence of rubber, and in fact the latex of many temperate Euphobias and Compositae contain triterpenol esters rather than rubber.

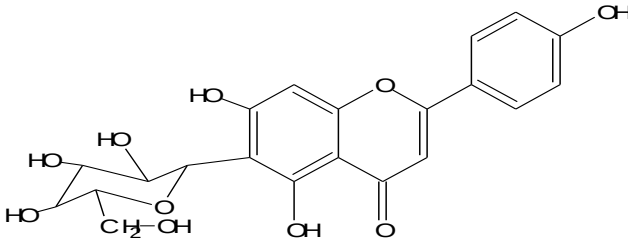
1.2 Phytochemical constituents of the Euphorbiaceae.

The chemical constituents of the Euphobiacea are important chemotaxonomic materials in the nomenclature of these medicnally important plants. The principal

constituents in Euphorbiacea are flavonoids which include vitexin and isovitexin (figure 6) (Galvez et al, 1993). These were isolated from the sap, leaves, stem and fruits. Triterpenoids are found in several genera of Euphorbiaceae. Latex of Euphorbiaceae usually contains phorbol, diterpenes, and/or daphnane esters (Figure 7, 8). Phorbol esters are known to be carcinogenic and are irritant (Gundidza, et al, 1993) and exists in both active and inactive forms in the plants. Symptoms can include pain and blistering, contact with the esters may result in temporary blindness (Gundidza,1992). Anthocyanins were detected in clustia such as dephinidin and cyanidin were also detected (Fig.9). Alkaloids (principally peptidic alkaloids) are commonly found in these plants, Hymenocardine a peptide alkaloid was isolated from *Hymenocardia acida* Tul (Pais et al, 1968). Other constituents include: Proteolytic enzymes, Mustard oils are present in Drypetes and putranijiva. (Irvine ,1961).



Vitexin



Isovitexin

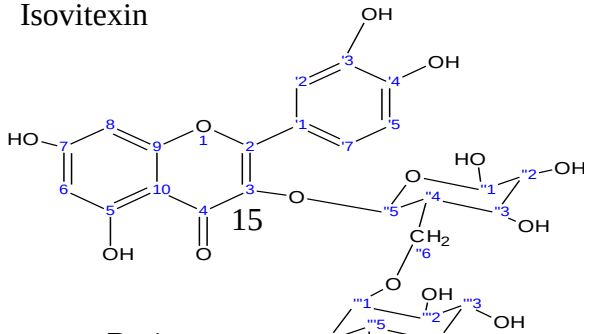
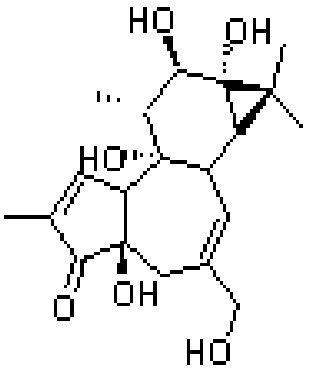
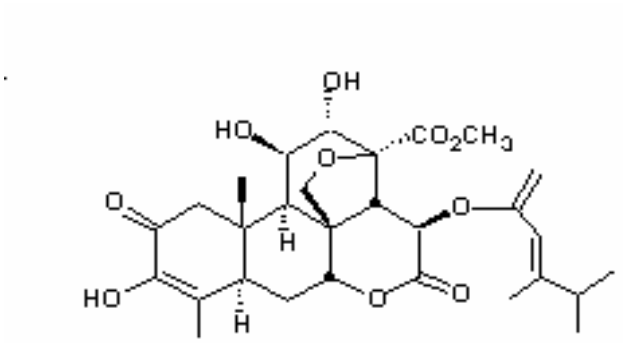


Figure 6:Flavonoids types of the Euphorbiaceae



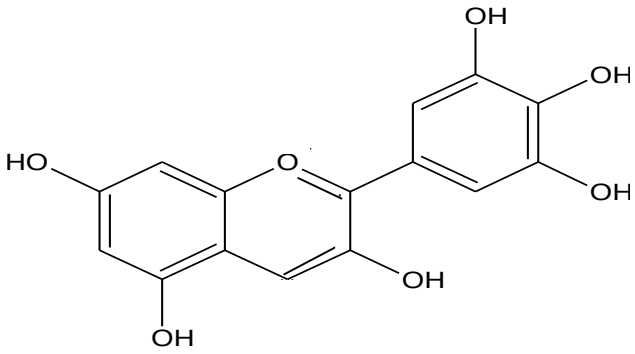
phorbol

Fig. 7: Phorbol of the Euphorbiaceae

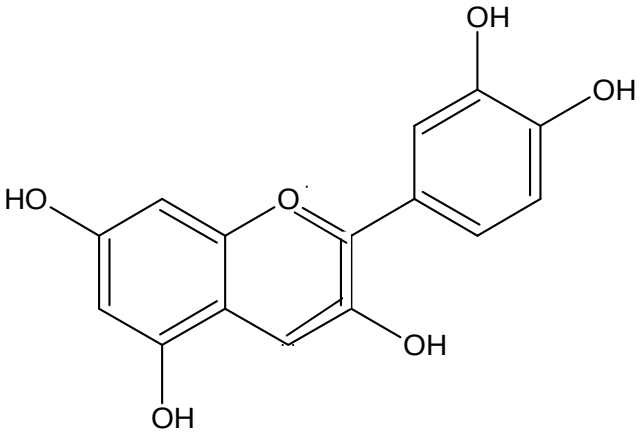


bruceantin

Fig. 8: Dipterpenes from Euphobiaceae



Delphinidin



Cyanidin

Fig 9: **Anthocyanins of the Euphorbiaceae**

1.3 Economical and medicinal uses of the family Euphorbiaceae

Plants in the Euphorbiaceae family have several economic importance. Several latex product are of commercial value, example chile obtained from *Achras sapota* (Sapotaceae) is used in making chewing gum. Opium is obtained from the latex of *Papaver somniferum* (Fahn, 1979) and is utilized in making cough syrup, has centrally acting analgesic activity and is used for recreational purposes. Some plants yield edible roots and fruits. The starchy roots of *Manihot* are used as food and commercial starch used as binders and diluents in tablet formulations (Irvine, 1961). The fruits of *Aleurites moluecana, Bridelia* and *Trevia* are used for culinary purposes. *Baliospermum micranthum* is used as vegetable. Fruits of *Emblica officinalis* are also edible.

Some plants are medicinally valuable. Castor oil is used as a lubricant and purgative. Croton oil is also used as a purgative while the milky juice of many plants such *as Manihot, Buxus, Euphorbia* is a potent poison and is used as insecticide.

Wood oil, drying oil etc. are extracted from the seeds of several species. *Hevea brasilensis* and *Manihot glaziovii* are commonly cultivated is South India for the extraction of the latex. The bark of *Bischofia javanica* is useful in tanning. *Sapium* yields an important dye. Many representative species are used as ornamentals, especially *Euphorbia splendense, E. pulcharima, Ricinus, Codiaeum variegatum* (Crotan), J*atropha* and *Acalypha.* (Watson and Dallwitz 1992).

The irritant latex has an inflammatory property, it is co-carcinogenic, tuber promoter (Evans *et al*, 1975). In native medication, the non-irritant is used in the treatment of warts and tumour (Hartwell *et al*, 1969).

1.4 Description of the genus Hymenocardia Wall

These are trees, or shrubs; leaves are deciduous, alternate, shortly petiolate, simple, lamina entire; pinnately veined, or palmitately veined. Leaves stipulate, lamina margins entire. The flattened two winged fruits are unmistakable, separating from the persistent central axis; seeds parsely endospermic flat. Flowers aggregated in inflorescences, in racemes, or in spikes or in catkins. Flowers regular, 4 - 6 cyclic free hypanthum absent.

There are no petals and the stalkless male flowers are closely arranged with inconspicuous bracts and have 4-6 very short stamens with anthers free of one another, pollens shed as single grains. The females are borne in short leafy racemes; the two slender unbranched persistent styles are distinctive (long and free) gynoecium two carpelled. The pistil two celled, ovary two locular (compressed at right angles to the plane of the septum). Placentation apical. Ovules two per locule, pendulous anatropus (Watson and Dallwitz, 1991).

1.5 Description of *Hymenocardia acida* Tul

The Local names are Jan Yaro (Hausa) (Gbile, 1980),Yawa Satoje (Fulfulde), Ikalaga (Igbo), Orupa (Yoruba).

The plant is widely distributed in tropical Africa, mostly found in Senegal, S. Leone, Togo and Nigeria. It is found mainly in the savanna forest. It is a shrub or tree usually less than 4.5m high but sometimes attaining as much as 9m. Bark gray-brown to rusty orange. Leaves pubescent when young, becoming glaborous or nearly so;

densely beset with golden glands beneath, elliptic – oblong, up to 8.75cm in length and 3.75cm broad, apex obtuse to rounded, base obtuse, petiole slender, up to 1.9cm long; calyx cupular, red; anthers creamy white. Female flower axillary on leafy lateral branchletes, calyx divided to the base; styles 2 crimson, spreading about 1.25cm length. Fruit compressed, winged, obcordate, 2.5cm long and 2.5cm – 3.5cm broad. Wood pale brown, darkening on exposure to orange, showing well defined growth rings, very hard, very dense, very durable, highly resistant to termites. (Brandis, 1971).

1.6 Description of other *Hymenocardia* species

The genus *Hymenocardia* consists of five species which include:

1.6.1 *Hymenocardia heudelotti* Muell (syn. *H. cheralien* Beille)

This is found in Savanna and rainforest of West Africa mostly distributed in Senegal, Guinea and Nigeria. The plant grows along the banks of streams in forest, example, Northern and Eastern Nigeria.

It is a shrub or small tree up to 9m high, branchlets very dense, short and pubescent. Leaves are up to 4 x 5 – 7.5cm, elliptic to broadly oval or rounded, cordate at base, obtuse at beneath, hairy on the midrib and nerves but otherwise glabrous with minute glandular dots on the under surface.

Male spikes 2.5 – 5.0cm long. Fruits 2.5 – 3.0cm broad, 1.4-1.6cm long, reticulate, glaborous or pubescent, brown without two terminal wings.

1.6.2 *Hymenocardia lyrata* Tul.

Found in closed forest and dry transition forest near edge of Savanna. Distributed along Senegal to Ghana.

This is a shrub or tree up to 15m high and 1.5m wide. The bark is dry and pale brown. Sucker shoots are frequent on the stem and the leaves are over 3 times 5cm, ovate – elliptical, long or acuminate apex, base is rounded, papery 3.5 – 8.5cm long, 1 – 4cm broad. Axis of the sinus, the wings well developed and ascending. Wings of the cocci almost touching at the top, forming a very narrow sinus, fruits lyrate, 2.0 – 2.5m long; forest species. (Dalziel, 1937, Keay, 1964)

1.6.3 *Hymenocardia intermedia* Dinkl.

This is an imperfectly known species possibly *H. lyrata* or a hybrid.

1.6.4 *Hymenocardia pollis*.

This is probably only a pilose form of *H. acida.*

1.7 Medicinal uses of *Hymenocardia acida*

The young fruits and shoots are acidic in taste and are occasionally eaten (Irvine, 1961). Decoction of the bark is used in Guinea-Bissau and Ivory Coast as an aphrodisiac and as a remedy for sleeping sickness (Kerharo and Banquet ,1950;Kerharo, 1974,; Bouquet and Debray, 1974). Entire part of this plant is used against fatigue in Nigeria (Vasileva, 1969). The root decoction is used also in Nigeria for fever, it is also used in Ivory Coast as aphrodisiac and for conjunctivitis, trachoma, and it is also used as a febrifuge in Ashanti.

It is used as Galactogogue (Bouquet and Debray, 1974). An infusion of the bark and leaves is considered effective in Guinea for the treatment of respiratory tract infections. The leafy stem and bark are used for bathing and lotion to strengthen sick children and as a beverage for diarrhoea and dysentery (Irvine, 1961).

The charred twigs are rubbed for headache and the powdered bark with lime for colds (Irvine, 1961). The powdered bark alone as a decoction is considered as antiseptic and is used in Nigeria to wash wounds and ulcers while a decoction of pulverized bark is used internally for colic.

The leaves have numerous medicinal properties. In Guinea, the leafy decoction is used for biliousness. In Ivory coast, the leaf decoction used as a beverage or bathing is a well-known febrifuge. The powdered leaves are used as snuff or applied locally for well-known febrifuge. The powdered leaves are used as snuff or applied locally for various pains, example headaches, rheumatic pains and toothaches where they are crushed with lemon juice (Irvine, 1961). The plant is used in Northern Nigeria as an ingredient in a

complex Hausa prescription to give strength on a journey (Dalziel, 1937), while the leaves and stem-bark are used in the treatment of eye infection and sickle-cell anaemia.

The dried leaves is used in Zaire for Diarrhoea, also used for chest pains, coughs, anaemia, urinary tract infections, infected wounds, for skin diseases, diabetes, and purulent sores. The dried root bark is also used in Zaire to treat dental caries, sterility, diarrhoea and chest pains (Muanza et al, 1994). The dried root of the plant is used to treat purulent sores and is also effective against vibrio cholera (Silva et al, 1996). Boiled bark and leaves of *H. acida* with those of pterocarpus santalinoides and pseudarthria hooker, the decoction taken in large quantities, two times a day for nine days is used for the treatment of sleeping sickness (Kerharo, 1974).

The plant is also reported to have the following pharmacological actions: The aqueous stem-bark extract of *H. acida* is found to be effective as an anti-ulcer (Ukwe, 1997). In Senegal the dried stem-bark is also found to be very active as an antimicrobial, antibacterial, antifungal, anti-tumour. (Muanza et al,1994; Muanza et al,1995). It was found not to be active against human immune virus (HIV) (Silver *et al,*1996) Other reported biological activities of the plant include antispasmodic and anti-diarrheic activity (Kambu *et al*, 1990).

1.8 Economic uses *Hymenocardia acida*

The bark is also used in Chad region and central Africa for tanning leather. The wood is said to be resistant to termites and it is made into pestles and bark-cloth mallets in Uganda. The bark is used as a red-brown dye for raffia (Irvine, 1961).

1.9 Phytochemistry

Phytochemistry is the study of chemical constituents present in plants. The plants constituents are usually classified into two groups, the primary metabolites such as carbohydrates, proteins and lipids and secondary metabolites such as glycosides, alkaloids tannin, terpenes, steroids, rasins and balsams. The primary metabolites do not have pharmacological activity but may be of pharmaceutical necessity while the secondary metabolites have pharmacological activity.

1.9.1 Carbohydrates:

The carbohydrates are compounds consisting of carbon, hydrogen and oxygen, the two elements being usually present in the same proportions as in water. They are among the most abundant constituents of both plants and animals. Many such as the sugars and starches, are important food reserves for the plant and foodstuffs. Cellulose and other polysaccharides are constituents of cell walls of plants. Of special pharmaceutical importance is the fact that sugars unite with wide variety of other components to form glycosides.

The carbohydrates are generally water soluble, the sugars have a sweet taste while the more complex ones are tasteless. The solubility decreases with increase in complexity.

The carbohydrates usually classified into the monosacchrides are made up of one sugar molecule of 3 – 9 carbon atoms. Those of five and six carbon atoms (pentoses and hexoses respectively) are the most abundant in plants. The oligosaccharides are made up

of two to nine sugar molecules. The polysaccharides consist of more than nine sugar molecules. (Evans, 1996)

1.9.2 Tannins

The term ‘tannin’ denote substances present in plant extracts which are able to combine with protein of animal hides, prevent their putrefaction and convert them into leather. From the above definition, tannin is a substance which is detected qualitatively by a tanning test (the gold-beater’s skin test) and is determined quantitatively by its absorption on standard hide powder. This definition excludes simpler phenolic substances, often present with tannins such as gallic acid, catechins and chlorogenic acid, although under certain conditions they may give precipitates with gelatin and be partly retained by hide powder. Such substances of relatively low molecular weight are called ‘psuedotannins’. Most true tannins have molecular weights of from 1000 to 5000. These true tannins are classified into hydrolyzable tannins and condensed tannins (Evans ,1989).

1.9.3 Glycosides

Glycosides are complex compounds which yield upon hydrolysis one or more sugars (glycone) and one or more other products (aglycone). Glycosides have many functions. In plants they serve as sugar reserves, as waste products ofs metabolism, as a means of detoxification, to regulate osmosis and to stabilise labile substances of importance in metabolism (McIlory, 1951).

The glycosides are normally linked between the glycone and aglycone through oxygen. These form of glycosides are known as glycosides. They are easily hydrolysed to the parent sugars and the aglycone by either enzymes or acids. The sugar moiety can exist either in $\propto$ or β forms, thereby yielding both $\propto$ or β glycosides. The sugar oxygen ring is retained and the sugar can occur in the pyranose or furanose firm. The former is the most common.

If the reducing group of the sugar is linked to a thiol, S-glycosides are obtained.

Present in plants also are N-glycosides which involves linkage to an amino group such as the nucleosides from ribose and purines, vicine and crotonoside.

Several glycosides are known in which the sugar moiety is not a true sugar, but a sugar derivative such as uronic acid.

Included in the consideration of glycosides is a group of compounds known as C-glycosides, which resist normal acid hydrolysis but have infra red spectra, and yield alkaline degradation product which indicate the presence of a sugar-link chain (Miller, 1973).

The different types of glycosides include:

- Cyanogenic glycosides
- Anthraquinones.
- Flavonoids
- Saponins
- Cardiac glycosides

Cyanogenic glycosides - are those glycosides that liberate hydrocyanic acid among the products of hydrolysis. Many of these glycosides, but not all, are derived from the nitrile

of mandelic acid. Although they contain nitrogen, their structure is that of 0 – and not N-glycosides (Evans, 1996).

Anthraquinones - are glycosides consisting of anthraquinone nucleus or its derivative; the anthrone and anthranols, the oxanthrones or the dimers of these compounds. Bontrager's test is usually used for their identification (Evans, 1996).

Flavonoids - which occur in both the free and as glycosides are the largest group of naturally occurring phenols. The flavonoids have a basic nucleus of the chromone (benzo-γ-pyrone). They may occur in different forms such as the flavones, isoflavones, flavonols, flavanones, chalcones or xanthones, most are O-glycosides and may occur as dimeric compounds (biflavonols). Flavonoids dissolve in alkalios giving yellow solutions which on the addition of acid became colourless (Evans, 1996). The flavonoids are important to man not only because they contribute to plant colour but because many members (e.g. coumestrol, phleridin, rotenone) are physiologically active (Harborne *et al*, 1975).

Saponins - are glycosides characterised by their property of producing a frothing aqueous solution. They have haemolytic properties. The fact that a plant contains haemolytic substances is not a proof that it contains saponins. When taken by mouth saponins are comparatively harmless.

Saponins have high molecular weight and their isolation in a state of purity presents some difficulties. As glycosides they are hydrolysed by acids to give an aglycone (sapogenin) and various sugars and related uronic acids. According to the structure of the aglycone two types of saponins are recognised, the steroidal and

triterpenoidal. Both of these have a glycosidal linkage at C-3 and have a common biogenic origin via mevalonic acid and isoprenoid units (Evans, 1996).

Cardiac glycosides - are those glycosides, which therapeutically strengthen a weakened heart. The therapeutic efficiency depend both on the structure of the genin and the type and number of sugar units to which it is attached. There are two types of cardiac glycosides depending on whether the genin has five or six numbered lactone ring. These types are known as cardenolides and bafanolides or bufadienolides (Evans, 1996).

Generally, the glycosides are soluble in aqueous solvent while the aglycones are soluble in non-polar solvents.

1.9.4 Resins and balsams

The term resin is applied to more or less solid, amorphous products of complex mixtures of resin acid, resin alcohols (resinols), resin phenols (resinotannols), ester and chemically inert compounds known as resenes. Rasins are often associated with volatile oils and gum (Oleo-gum-resins).

Balsam is used to denote substances containing a high proportion of aromatic balsamic acids (Evans, 1996).

1.9.5 Alkaloids

A precise definition of the term alkaloid (alkali-like) is somewhat difficult because there is no clear-cut boundary between alkaloids and naturally occurring complex amines. Typical alkaloids are derived from plant sources. They are basic,

contain one or more nitrogen atoms (usually in heterocyclic ring) and usually have a marked physiological action on man or other animals. (Evans, 1996)

1.10 Stems-bark and leaves

1.10.1 Stem-bark

The stem is an ascending axis of the plant developed from the plumule, it consists of nodes, internodes and buds and it gives rise to branches, leaves and flowers. The stem may be aerial, subaerial and underground. Depending upon the presence of mechanical tissues, the stems may be weak, harbaceous or woody. The Bark is the secondary external tissue lying outside the cambium in stem or root of dicotyledonous plants, is known as the bark botanically, bark is also known as periderm consists of three layers viz., cork (phellem) cork-cambium (phellogen) and secondary cortex (phelloderm).

Commercially barks consist of all the tissues outside the cambium. A young bark includes epidermis, cortex, pericycle and phloem. Barks are obtained from the plants by making longitudinal and transverse incisions through the outer layers followed by peeling. Barks may be obtained from stems or roots. Due to the excessive growth produced by the cambium and cork cambium, the external tissues get tangentially stretched or torn and hence, the epidermis is not found in the barks.

a) **Characteristics of barks**

Barks exhibit several morphological and microscopical characters. The morphorlogical characters need special attention, as they help in identification of the barks.

b) Shapes in barks

The shape or form of the bark is dependent upon the method adopted for its preparation. It also depends on the type of incision made and the extent of any subsequent shrinkage of the tissues. When the bark is removed from the large trees and dried under pressure, the flats are produced e.g. quillaia and arjuna. When the bark is removed from the small branches due to shrinkage of the soft tissues it tends to curve forming concavity on the inner side. Yielding curved pieces e.g. wild cherry and cassia.

If concavity is on the outer side of the bark, it is described as recurved e.g. kurchi when the shrinkage of the tissues is to a greater extent and it forms deep trough or channel. It is called a channel bark e.g. ashoka, Cinchonna ledgeriana and Cassia in some cases, one edge of a bark covers the other to form quill e.g. cascara and cinnamon. If both the edges of the bark roll independently forming quill, it is described as double quill e.g. Java cinnamon. In some cases, one quill of a bark is put inside other quill to form a compound quill e.g. cinnamon compound quill is a man made shape of bark. It reduces the exposure of bark to atmospheric conditions and also saves the space in transport.

c) Fractures in bark

The appearance shown by the transversely broken surfaces of the bark is known as fracture. It is sometimes, useful in identification of barks. The types of fractures are as follows:

When fractured surface is smooth, it is described as a short fracture (cinnamon and kurchi). If the exposed surface exhibits small rounded appearance, it is described as a granular fracture (wild cherry and cassia). If the broken surface shows the presence of uneven projecting points, it is described as splintery fracture, as seen in cinnamon. The presence of numerous fibres on the transversely broken surface is described as a fibrous fracture (cinchona). If the exposed surface shows the arrangements of layers one over the other, it is described as a laminated fracture, as observed in quillaia.

The various characters shown by the barks on the outer, as well as, inner surface are also diagnostically important. Amongst these, the colour, condition and presence of several growths like lichens, mosses etc, are characteristics to each bark. The presence of lenticels and development of cracks are additional characters of bark. Outer surface of the bark shows the presence of cracks and fissures, which are due to lack of elasticity or due to increase in girth of the trees. Fissures are usually deep, wrinkle which are seen on outer surface of the bark, result due to shrinkage of inside soft tissues. Furrows are troughs between wrinkles. Inner surface of bark shows

characteristics such as striations, which are longitudinal and parallel lines. Transverse wrinkles present on inner surface are described as corrugations.

1.10.2 Leaves

Leaves are flat, thin, and green appendages to the stem, containing, supporting and conducting strands in their structures. In pharmacognosy, the word leaf includes leaf, compound leaf and leaflets. Usually the minimum percentage of active constituents is specified in leaves.

Apart from the normal characteristics i.e. their arrangements on the stems, their apices, margin, petiole, presence or absence of stipules etc. leaves are characterised by certain diagnostic structures. Most of these diagnostic characters are microscopic one such as stomata and trichomes.

1.11 Quantitative evaluation of crude vegetable drugs

The evaluation of a drug requires that it be identified both in its entire and powdered form by means of its morphological and cytomorphological characters. It is possible that faulty collection or extended storage may alter the constituents of the drug to a part where it is no longer acceptable. Therefore, drugs are subjected to evaluative procedures which will indicate their acceptability by criteria other than their morphology. (Brain and Turner, 1975).

The different determinations of quantitative analysis of crude vegetable drugs include the followings:

1.11.1 Determination of moisture content:

The determination of moisture content is important because the presence of moisture causes the following:-

- It decreases the percentage quantity of the drug by increasing the weight.
- Causes activation of enzymes which lead to hydrolysis of the constituents.
- Causes chemical reactions and
- Harbours the growth of micro-organisms and insects.

Therefore the moisture content of all drugs should be kept reasonably low (Shellard, 1958)

1.11.2 Determination of ash value

The determination of total ash is a method to measure the amount of residual substance not volatalised when the drug is ignited (African pharmacopoeia, 1986). The total ash represent the 'physiological ash' (i.e. ash that comes from the plant tissue itself) and non 'physiological ash' (i.e. ash from the extraneous matter (African Pharmacopeia, 1986). The physiological ash consist of the carbonates, phosphates, nitrates, sulphates, chlorides and silicates of the various metals present in the plant which it took up from the soil when growing. Example of elements taken up by the plant from the soil include sodium, potassium, calcium, magnesium,

iron, copper, cobalt, manganese, carbon, phosphorous, nitrogen, sulphur, oxygen, chlorine and silicon (Shellard, 1958).

1.11.3 Determination of extractives

The determination of extractive is a method designed to measure the amount of constituents which are extractable by the solvent under specific conditions. It is applied to those vegetable drugs for which there is no suitable method of chemical or biological assay for their active constituents (African Pharmacopoeia, 1986). The evaluation is important to guard against adulteration or substitution with drugs which have already been extracted (Shellard, 1958).

1.12 Chromatography

Among the various methods of separating plant constituents, the chromatographic procedure originated by Tswett is one of the most commonly used techniques of general application.

In 1944, Consden, Gordon and Martin introduced a method of partition chromatography using strip of filter paper for the analysis of amino acid mixture.

Chromatography represents a group of methods for separating molecular mixtures that depend on the differential affinities of the solute

between two immiscible phases. One of the phase is a fixed bed of large surface area which is called a stationary phase, while the other is a fluid or gas which moves through or over the surface which is called the mobile phase. The stationary phase may be a porous or finely divided solid or liquid that has been coated as thin layer on an inert support material. The mobile phase may be a pure liquid or a mixture of solutions or it may be a gas or mixture or gases.

For the separation of some bio-constituents, it is necessary to use a two-dimensional chromatography using two different solvent systems. The resolved components of original mixture can be separately eluted from chromatogram by treating the cutout spots with a suitable solvent and then determined quantitatively by some suitable instrumental method of analysis, for example fluorescence analysis, colorimetry, or ultra-violet absorption .

1.12.1Thin layer chromatography (TLC)

In 1958, Stahl, demonstrated application of TLC in analysis, a method based on adsorption chromatography. It is at present an important analytical tool for qualitative and quantitative analysis of a number of natural products. The adsorbent such as silica gel G. or C is coated to a thickness of 0.3mm on clean TLC plates using commercial spreader, the plates are activated at 105^0

for 30 minutes and used. The selection of mobile phase depends upon type of constituents to be analysed. After the development of chromatogram by ascending technique, the resolved spots are revealed by spraying with suitable detecting agent. TLC has certain advantages over paper chromatography. Fractions can be effected more rapidly with smaller quantities of mixture, spots are usually more compact and drastic reagent, such as concentrated sulphuric acid can be used as spraying solutions. The TLC techniques is useful in analysis of alkaloids, glycosides, isoprenoids, lipid components, sugars and derivatives and practically all bio-constituents. The Rf values (distance travelled by component / solvent front) may vary depending upon purity of solvent, nature of substance to be resolved, composition of solvent, presence of impurities, adsorbent used, polarity of the solvent, substance and absorbent etc.

TLC and HPTLC techniques, nowadays, are important analytical tools for micro-analytical separation and determination of natural products. They have following advantages.

- **simple to operate, economical, rapid (particularly HPTLC)**
- **always available for use (precoated plates are commercially available)**
- **method of detection does not place any restriction on choice of eluent, easily inspected**

- **neutral, basic, acidic or purely aqueous eluents can be employed. The whole chromatographic system is flexible.**

TLC can be exploited in the investigation and cultivation of medical plants. It is possible to run many samples of extracts from different chemical races simultaneously with authentic standards and high performance individuals can be recognized, selected and bred. This technique is also valuable to discovery of so-called chemical races (Priti and Misra, 1994).

1.12.2Column chromatography

Basically, it is a liquid chromatography in which mobile phase in the form of liquid passes over the stationary phase packed in a column. The column is either a glass or metallic column. The column adsorption chromatography is the oldest one and still practised today for extraction purposes. Column chromatography has been derived into other forms like gel permeation, ion exchange, affinity and column partition.

In the column adsorption chromatography, fairly large number of adsorbents are used like starch, calcium carbonate, magnesia, lime, silica gel, alumina, charcoal and fueller's earth. To optimise the resolution, various mobile phases used, either singly or in combination, are petroleum ether, cyclohexane, chloroform, acetone, water, pyridine and organic acids. For the better separation, it

is essential to consider the polarity of the sample, adsorbent and mobile phase (Pritis and Misra, 1994).

1.13 Biological studies

In standardization/evaluation of herbal drugs, assessment of biological efficacy is found to be most assuming method. However, there are protocols to be followed for assessment of some types of biological activities.

In all these methods, requirements are a suitable model for testing and control methodology for experiment and assessment of results, (Priti, *et al,* 1994).

1.13.1 Toxicology

Toxicology is concerned both with the nature and mechanisms of toxic lesions and quantitative evaluation of the spectrum of biological changes produced by exposure to chemicals (drugs). Every chemical (drug) is toxic under certain conditions of exposure (WHO, 1978).

Toxicity

Toxicity of a substance could be defined as the capacity to cause injury to a living organism (Sanockij, 1970).

A highly toxic substance will damage an organism if administered in very small amounts; a substance of low toxicity will not produce an effect unless the amount is very large. Thus toxicity cannot be defined without reference to the quantity of a substance

administered or absorbed (dose), the way in which this quantity is administered (e.g. inhalation, ingestion, injection) and distributed in time (e.g. single dose, repeated doses), the type and severity of injury, and the time needed to produce that injury.

Priorities in the selection of chemicals for testing:

In principle, all new chemicals (drugs) require safety evaluation before manufacture and sale, but because of the large number of chemicals (drugs) that represent a possible hazard to human health and limited resources it is necessary to give priority to those that are directly consumed by man, such as drugs and food additives, and those that are widely used such as pesticides or household consumer products. Industrial chemicals that can escape into the working or general environment or can contaminate other products are other category of concern.

Several types of toxicity testing procedures have been developed. These include acute, subacute and chronic studies. The major difference between these tests is the dose employed and the length of exposure to the drug.

All of the tests share some common characteristics each requires that groups of healthy animals, housed under suitable conditions, be exposed to graded doses of the test drug. Rats, mice, guinea pigs, rabbits and harmsters are commonly used for this purpose.

The controls and treated animals should be of the same strain and species, age and weight range, and be supplied from the same source. Before starting the experiment, the health status of all animals should be determined and maintained for sometime.

During toxicity studies, rodents should be housed singly or in pairs in stainless steel or plastic box cages.

The diet fed to the animals should meet all of their nutritional requirements and should be free of toxic chemical impurities that might influence the outcome of the test (National Academy of Sciences, 1975).

Although commercially available diets of recognized quality are suitable for most subacute studies, semi purified diets may be preferred because the nutrient and non nutrient components of the diet may be altered readily where necessary (Munro, 1974).

In toxicity studies many animals may be lost for evaluation because of death from intercurrent disease and subsequent autolysis, these loses can be greatly reduced if conscientious effort is made to recognize early chemical signs of disease in the test animal.

Detailed chemical examinations should be conducted weekly on the test animals by competent, laboratory animal technicians under the supervision of veterinarian skilled in laboratory animal medicine (Health and Welfare Canada, 1973). These should include general observation of the animals for overt signs of toxicity, quality of hair, coat, general conditions of the eyes, mouth, teeth, nose and ears (Leclair & Willard, 1970)

(a) Acute toxicity tests

Acute toxicity has been defined as the adverse effects occurring with in a short time of administration of single dose or multiple doses given within 24hrs (Hagan, 1959). When data are unavailable concerning the toxicity of the test agent, acute toxicity studies are indicated to identify the relative toxicity of the compound, to investigate its mode of action and its specific toxic effect and to determine the existence of species differences.

The most frequently used acute toxicity test involves determination of the median Lethal dose (LD_{50}) of the compound. The LD_{50} has been defined as "a statistically derived expressions of a single dose of a material that can be expected to kill 50% of the animals (Gehring, *et al*, 1973). The basic protocol for the determination of the LD_{50} is well established and consists of treating groups of animals with a mathematically – related series of doses in order to determine the dose that kills 50% of the group and the dose response function. Death that occurs after the first 24 hours is more likely to be due to delayed toxic effects, which may be direct or indirect. Signs occurring after the first 24 hours period may give some indication of the effect that the chemical may have at lower levels when administered for longer time periods.

1) Selection of species

The mouse, rat and dog are the most commonly used species for acute toxicity testing. Both rat and mouse should be used, as marked differences in the LD_{50} between these two species are not uncommon (Marrison *et al*, 1968).

The LD_{50} determination should be conducted in both male and female animals as differences in the LD_{50} between sexes have been well documented (Rumke, 1964) and probably related, in part, to differences in heptatic metabolism (Conney *et al*, 1965).

Acute toxicity may vary substantially with age of the test animal (Dieke & Richter, 1945) and animals of various age should be used in LD_{50} determinations. The effect of the age of the animal on the LD_{50} is well documented and may be related to different levels of drugs metabolizing enzymes, absence of sex hormonal

influences, or an altered sensitivity of the central nervous system (Fouts & Hant, 1965).

The animals should be derived from previously untreated healthy females.

The animals should not have been previously used for other studies, nor should there be history of recent exposure to antihelmintics or any other drug treatment.

The number of animals used should be sufficient for statistical analysis and will depend on the method used for the calculation of the LD_{50}. Usually 8-10 rodents (4-6 animals of each sex) are used per dose group (Leclair & Willard, 1970). Other methods involved the use of 4-5 animals per dose group (Horn, 1956).

2) Selection of doses

The doses are selected to provide data for estimating the LD_{50} at least four doses should be used (Weil, 1952).

The initial dose may be such that no effect is manifested in the animals. In subsequent groups of animals, the dose should be increased by a constant multiple until the dose of the compound (plant) administered is sufficiently high that all of the animals in the group die.

Under these conditions, data can be obtained that can be plotted to give a dose response curve and from which the LD_{50} value may be calculated.

3) Method of administration

Generally the chemical should be administered by the route which man would be exposed.

4) Postmortem examination

In general, all animals dying during the observation period and all surviving animals should be autopsied by a qualified pathologist (Leclair & Willard, 1970). The autopsy should include gross and histopathological examinations of all organs.

b) Subacute and chronic toxicity test

The subacute toxicity test generally involves daily or frequent exposure to the compound over a period up to about 90 days. It provides information on the major toxic effects of the test compound and the target organs affected (Barnes, 1960).

Acute and subacute toxicity tests are of limited value in predicting chronic toxic effects because (a) chemicals may produce toxic responses when administered repeatedly over a period and (b) during the aging process, factors such as altered tissue sensitivity changing metabolic and physiological capability and spontaneous disease may influence the degree and nature of toxic response. In addition, several important diseases such as heart disease, chronic renal failure and neoplasia are associated with advancing age.

Chronic toxicity tests, in which animals are exposed for their entire life time to environmental chemicals, have provided useful means of identifying those substances of greatest public health concern.

1) Experimental design

Selection of species and duration of studies:

In chronic toxicity testing it is usual to expose the animals to the chemical for the greater part of the life span. A wide variety of animals species have been used in this type of work, although in most cases rodents are the animals of choice since large animals should also be used to aid in the statistical interpretation of the result.

2) Method of administration

The rate of administration in chronic studies should be that through which man is likely to be exposed. For food additives, pesticides and other chemicals likely to come into contact with food or water, the oral route is recommended (Leclair & Willard, 1970).

The concentration of the test chemical in the diet should be determined periodically to ensure uniform dispersion and to aid in the qualification of achieved doses.

The diet is the preferred vehicle of administration, but it is absolutely essential that the chemical be present in the diet in an unaltered form; toxicity may be altered by interaction with dietery constituents (Kello & Kostial, 1973). In rodent studies, the

compound may be administered in the diet as a fraction of the total diet, or a sufficient quantity of the chemical may be added to the diet to achieve predetermined dose levels (in mg per kg body weight per day). In the later case, it is necessary to adjust the dietary concentration weekly or biweekly to maintain a constant dose level, since food consumption per unit of body weight decreases, as the animal gets older.

Food consumption and body weight should be recorded weekly in all animals weight gain per unit of food consumed should be calculated (Munro, 1969).

This gives a measure of the efficiency of food use. The daily dose of chemical or drug should be calculated form data on good intake and body weight.

The data can be used to estimate the dose of chemical received and are necessary in the establishment of dose – response relationships. Body weight changes serve as a sensitive indication of the General health status of test animals.

1.13.2 Haematology

This is a branch of medicine that deals with diseases of the blood and blood forming organs.

It is commonly divided into 3 sub-areas according to the type and group of cells it refers to:

1. **Anaemia**: Which is the state of having low hemoglobin levels. It is the most common disorder of the blood. When anemia is severe, the blood cannot transport sufficient amounts of oxygen, because the number of red blood cells is too low or something is wrong with the hemoglobin (the oxygen-carrying protein in the red blood cells). The tissues of a severely anemic person become, in a

sense, “starved” of oxygen. Deprived of oxygen, the tissues cannot produce enough energy to function properly. The most common physical complaint of people with anemia is a feeling of weakness or fatigue.

2. **Haemoglobinopathies**:

3. Blood bankings

1.13.3 Analgesic activity

Analgesic: means a medicine used in the relieve of pain or capable of relieving pain, the anodyne properties of certain drugs means the analgesic effect.

Pain: The word pain is derived from the Greek for penalty. Pain cannot adequately be defined in words. To overcome this problem one asks operative questions, example:

(1) What is the purpose of pain? It is a warning of tissue damage and its persistent nature has a protective function by ensuring that the subject cannot ignore it except with considerable voluntary effort.

(2) Is pain a universal experience? Complete congenital insensitivity to pain is very rare and apart from these few subjects with this condition, it is known from childhood what is meant by pain. However, the highly subjective nature of pain makes it difficult for one to communicate about it verbally; and then the words used reflect cultural, educational, emotional and other values.

(3) Does intense harmful stimulation always produce pain? The answer is very firmly, no, one has only to witness the astonishing denial of pain in a significant

proportion of major casualties, especially on the battle field, to realise that there can be on occasion no relation between trauma and pain. Whatever the explanation for this absence of pain it suggest that the body has its own pain controls which can be called upon to obtund pain in certain circumstances.

One can go on asking questions like these until a more complete mental picture of what is meant by pain is built up. (Hannington – Kiff, 1974).

Scientist from many disciplines have investigated pain and each group has used a definition suitable for that field. Terms used include pain, discomfort, suffering and nociception (the sense that damage is being doing done in some area of the body or the sense of something noxious). Pain is often listed as a sensation (Benjamin and Gorsky, 1981).

Measurement of pain threshold

There are three general methods used for assaying the effectiveness of an analgesic agent.

- Testing the effects of the agent on the reaction of an animal to noxious stimulation (thermal, electrical, chemical or mechanical stimuli).
- Measuring the pain-reducing effects in normal human subjects with experimentally induced pain.
- Measuring the pain reducing effects in patients experiencing pain spontaneously.

In general it may be said that animal testing serves as a rough assay of analgesia, test on normal human subjects offer a more precise assay of strength of analgesia, and

measurements in patients with pain evaluate the effectiveness of the agents under circumstances most relevant to clinical use (Balla and Bhargava, 1980; Dipalma, 1971).

1.14 Aims and objectives

The Hausa tribe of Northern Nigeria have claimed the usefulness of *Hymenocardia acida* in anaemia and in relieving pain (analgesic) but there are no published reports on the pharmacognostic, phytochemical and biological activity of this plant. The specific aims and objectives of this project are:

1. To study the general pharmacognostic and phytochemical profile of the stem-bark and leaves of the plant.
2. To carry out biological studies which include toxicity and analgesic studies

   This can provide a monograph on the plant and justify the used of the plant traditional medicine.

CHAPTER TWO

2.0 *MATERIALS AND METHODS*

2.1. Collection, Identification and Preparation Of Plant Material

Hymenocardia acida was collected in August 2000 from the bushes of Basawa Zaria, Nigeria.

The plant (plates 1 and 2) was identified in the field using keys and description given in the 'Flora of West Tropical Africa' (Hutchitson and Dalziel, 1963) and "Woody plants of Ghana" (Irvine 1961). The identity of the plant was confirmed at the Herbarium of Department of Biological Sciences, Ahmadu Bello University, Zaria.

The stem-bark of the plant was stripped off and the leaves were removed from their branches. Both stem bark and the leaves were air dried, powdered, sieved and stored in airtight containers until required for use.

2.2. Apparatus

Indicator paper

Beakers

Conical flask

Glass funnel

Measuring cylinder

Glass stirring rod & spatula

Hot air oven

Water bath

Separating funnel

Microscope

Slides and slide covers

TLC plate and TLC development tank

Weighing balance.

2.3. Reagents

The reagents used were all laboratory grade. These were

Alcohol

Acetone

Acetic anhydride

Barfoed's reagent

Chlor-zinc-iodine

Chloroform

Copper acetate

3, 5, Dinitro benzoic acid in 95% alcohol.

Dragendorff's reagent (Bismith potassium iodine).

Fehling's solution I (A- copper II oxide) and II (B – sodium hydroxide).

Ferric chloride

Formaldehyde

Glacial acetic acid

Hager's reagent (a saturated solution of picric acid)

Hydrochloric acid

N_{50} Iodine

Lead acetate (10% w/v in carbon dioxide tree water, BPC 1963).

Magnesium filings

Methanol

Mayer's reagent (potassium mercuric iodine)

Molisch's reagent (10% solution of α-naphthol in alcohol)

Phloroglucinol

Ruthenium red

Silica gel (for thin layer chromatography)

Sodium acetate

n- Butanol

Petroleum Ether (60-80^{O}C)

2.4 Macroscopical examination of the stem bark and leaves of *Hymenacardia acida*

2.4.1 Stem bark

The following features used for characterization of whole bark samples were observed and noted:

-origin, size, surface, colour, fracture, fractured surface, odour and taste.

2.4.2 Leaves

The following features were used for the macroscopical identification of the leaves:

Duration, leaf base, petiole, lamina, incision, shape, venation, margin, apex, surface, texture, colour, odour and taste.

(Evans, 1996).

2.5 Microscopical and Chemomicroscopical examination of stem bark and leaves of Hymenocardia acida.

2.5.1 Microscopical examination.

The powdered or macerated sample, the longitudinal and transverse section of the stem-bark and the leaves of *Hymenocardia acida* were used for these studies (Evans, 1996). Both qualitative and quantitative studies were carried out'

A small quantity of the powdered sample, macerated sample or a thin anatomical section was placed on clean slide. A few drops of chloralhydrate solution was added and covered with clean cover slip. The slide was heated over Bunsen burner flame for about 30 seconds, over heating was avoided as this would cause formation of chloral hydrate crystals leading to false observation When the tissue was sufficiently cleared , air bubbles if any were removed. A few drops of dilute glycerol were the added and the slide observed under compound microscope

2.5.2 Chemo-microscopical examination

The powdered samples and the different anatomical sections were treated separately on microscope slides and observed under the compound microscope for the presence of

phytochemical substances such as cellulose tannins gums and mucilage, fats and oils starch protein cutin suberin lignin and calcium oxalate crystals (Evans , 1996**).**

I Test for Lignin

The powered samples and anatomical sections of leaves, stem, stem bark and seeds were mounted in phloroglucinol and conc. hydrochloric acid. A red colour indicates the pressence of lignin.

II Test for Cellulose

The various samples were mounted in chlor-zinc-iodine; or N/50 iodine followed by 66% sulphuric acid. A blue colour indicates the presence of cellulose.

III .Test for Tannins

The samples were mounted in ferric chloride solution. A bluish or greenish colour indicates the presence of tannins.

IV. Test for Mucilages

The different samples were mounted separately in Ruthenium red. A red or dark pink colour indicates the presence of mucilages.

V. Test for Cutin and Suberin

When the different samples are stained with tincture of alkanna, a red colour indicate the presence of cutin and suberin.

VI .Test for Callose

The different samples were stained with corallin soda. Red colour indicates the presence of callose.

VII Test for Starch

The different samples were mounted separately in N/50 iodine. A blue colour indicates the presence of starch.

VIII Test for Calcium Oxalate Crystals

The samples were separately cleared in chloralhydrate solution. If calcium oxalate crystals are present, they are seen as bright structures of definite shapes and sizes. After addition of a few drops of conc. Sulphuric acid (80%) and reviewed under the microscope the disappearance of calcium oxalate crystals confirms their presence.

IX Test for Oils

The samples were separately mounted in Sudan IV reagent. A pink colour in any of the structures indicates the presence of oils.

X Test for Protein

1% Picric acid and Million's reagent were used. When the different samples were stained separately with picric acid a yellow stain on the microscopic structures indicates the presence of protein. A red stain with Million's reagent indicates the presence of protein.

2.5.3 Quantitative microscopical examination of the leaf of *Hymenocardia acida*

Quantitative microscopical values for the leaves were determined using standard methods. These numerical values include stomata number, stomata index, palisade ration, vein islet number, and vein termination number.

(i) **Determination of Stomata Number and Stomata Index**

To determine the stomata number and hence the stomata index of *H. acida* leaf; the leaf was cleared by heating in a test tube with chloral hydrate for about 10 - 15 minutes over water-bath and allowed to cool. After cooling the thin upper layer of cells of the lower epidermis was removed using a razor. It was then transferred to a microscope slide and mounted in a solution of chloral hydrate and a drop of glycerol and covered with cover-slip. Using a 4mm objective, a camera lucida, drawing paper and stage micrometer; a line was drawn on the paper equivalent to 1mm, as seen through the microscope. The mounted preparation was placed on the microscope stage and marked off, the stomata cells as (O) and the epidermal cells as (X), each enclosed in the square

drawn which is equivalent to 1mm. The number of epidermal cells (X) was counted and the stomata index was calculated using the formula: -

Stomatal index

$$I = \frac{S}{E+S} \times 100$$

S = No. of stomata per unit area

E = No. of ordinary epidermal cells in the same unit area

The procedure was repeated for 5 more fields and the average calculated.

(ii) **Determination of Palisade Ratio**

The fresh leaf of *H. acida* was boiled in choral hydrate for 10 - 20 minutes in a test - tube which was placed over the water bath. A thin transverse section of the leaf was removed with the aid of a razor and the teased section observed under the camera lucida. Four cells of the upper epidermis were traced off and the palisade cells under the epidermal cells were counted; completing the cells that cross the epidermal walls. The procedure was repeated three times taking different fields and the average noted, the palisade ratio was then calculated.

(iii) **Determination of Vein-Islet Number**

A portion of *H. acida* leaf was cleared and the lower epidermis of the uppermost was removed as described above. Using a 4mm objective lens, a camera lucida, drawing paper and stage micrometer a line was drawn; equivalent to 1mm as seen through the

microscope, a square was constructed on the line. The paper was moved so that the square was seen, on looking through the eye piece. The mounted preparation was placed on the microscope stage and traced. The veins enclosed in the square and those necessary to complete the islets that overlap the bottom and the left side of the square. From the drawing the islets were counted, including those that are intersected by the bottom and left side of the square; but excluding those inserted by the top and right side. Five different fields were viewed and the average is the vein-islet number.

(iv) **Determination of Vein-Termination Number**

The same procedure (iii) above was repeated: but the lower epidermis uppermost was removed from the leaf lamina to include the margin instead of between the midrib and the margin.

2.6 QUANTITATIVE EVALUATION OF CRUDE DRUGS

2.6.1 Determination of moisture content of the powdered stem bark and leaves by the "loss on drying method"

An evaporating dish was heated to a constant weight in an oven at 105^0C and its constant weight was noted. 3g of the powdered stem bark and leave was accurately weighed into the dish. The evaporating dish with its content was put in the oven at 105^0C and the content dried to a constant weight at 30 min interval after initial drying of one hour. Two consecutive same weights confirm a constant weight. The total loss in weight (i.e. the weight of the moisture) was determined by subtracting the constant weight of the dish and powdered stem bark and leave after heating from the weight of the dish and content before heating. The percentage of the moisture content with reference to the initial weight of the powdered stem bark and leave used was then calculated. This was done by dividing the weight of the

moisture by the weight of the drug taken multiplied by a hundred. Three different determinations were done and the average of these was taken as the moisture content of the drug (Evans, 1996).

2.6.2 Determination of Ash Values

(a) Determination of total ash

A nickel crucible was heated at 105^0C to a constant weight and its accurate weight was noted after cooling in the dessicator. 2g of each of the powdered stem bark and leave was accurately weighed into the crucible. The crucible and its content were gently heated over a microbunsen flame until it was moisture free and completely charred. The microbunsen flame was slightly increased until most of the carbon had volatalised and the inorganic ash was left. The ash was cooled, weighed and the weight noted. The heating and cooling was continued until the weight of the ash was constant. The weight of the ash was calculated by subtracting the weight of the crucible from the final weight by the weight of crucible and ash (B.P., 1980)

(b) Determination of acid insoluble ash value

The crucible with the ash from the experiment above was transferred into a beaker containing 25ml of dilute hydrochloric acid. The beaker and its content were boiled for 5 min and filtered through an ashless filter paper. The crucible and the beaker were washed with distilled water and the washings passed through the filter paper. The washings were repeated three times, every time the washings were

passed through the filter paper in a manner as to allow the collection of the residue at the tip of the cone of the filter paper. The funnel along with the filter paper was dried in the oven at 105^0C. The weight of a clean and heated nickel crucible was accurately determined using a sensitive balance. The filter paper with its residue was folded into a small cone and transferred into a crucible. The crucible was gently heated until the filter paper was completely ashed, then heated strongly for a few minutes. The crucible and its content were cooled, weighed and the final weight was noted. The weight of the residue (ash) was then calculated. This was done by subtracting the constant weight of the crucible from the constant weight of the crucible and its content (as). Also, the acid insoluble ash value with reference to the initial weight of the powdered stem bark and leave was calculated and expressed in percentage. The weight of ash divided by the initial weight of the drug multiplied by hundred was taken as the acid insoluble ash value (B.P. 1980).

2.6.3 Determination of Extractives

(a) Alcohol – soluble extractive value

5g of the powdered stem bark and leaves was accurately weighed into a 250ml stoppered conical flask. 100ml of 90% ethanol was added and the stopper was replaced firmly. The flask was shaken on a mechanical shaker for six hours and then allowed to stand for eighteen hours. The extract was filtered by suction filtration. The weight of a heated, cooled flat bottom evaporating dish was accurately determined. 20ml of the filtrate was taken into the weighed evaporating dish. The filtrate was evaporated to dryness. The residue was dried to a constant

weight at 105^{0}C in an oven and the final weight was noted. The weight of the residue obtained from 20ml extract was determined by subtracting the weight of the evaporating dish from the total weight of evaporating dish and the residue. The alcohol extractive value was then calculated with reference to the initial weight of the powdered drug and expressed in percentage (Brain and Turner, 1975).

(b) Water – soluble extractive value

The above experiment was repeated but using 0.25% chloroform water instead of 90% ethanol as the extracting solvent. The water soluble extractive value was calculated similarly as in the previous experiment (Brain and Turner, 1975).

2.7 Phytochemical Screening of Powdered Leaves, and Stem Bark of *Hymenocardia acida*

2.7.1 Test for carbohydrates

(a) Extraction

3g of the powdered samples were boiled separately in 50ml of distilled water on a hot plate for three minutes. Each mixture was filtered while hot and the resulting filtrate cooled and used for the following carbohydrate tests.

(b) **General test – Molisch's Test**

A few drops of Molisch's reagent was added to 2ml of each of the water extract obtained above, then a small quantity of conc. Sulphuric acid was allowed to form a lower layer. A purple ring at the interface of the liquids indicates the presence of carbohydrates.

Each mixture was then shaken and allowed to stand for 2 minutes and diluted with 5ml of water a purple precipitate also indicates the presence of carbohydrates (Evans, 1996).

(c) **Test for sugars**

Barfoed's Test – (General Test for Monosaccharide)

1ml of dilute solution of each sample of the water extract and 1ml of Barfoed's reagent were taken in a test tube and heated in a water bath for 2 minutes. A red precipitate indicates the presence of monosaccharide (Evans, 1996).

(d) **Fehling's test for free reducing sugars.**

To 2mls of each water extract was added 5mls of a mixture of equal volumes of Fehling's solutions I (A) and II (B) and boiled in a water bath for 2 minutes. A brick red colour indicates the presence of free reducing sugars (Evans, 1996).

(e) **Standard test for combined reducing sugars**

1ml of each of the water extract was hydrolysed by boiling with 5ml of dilute hydrochloric acid. This was neutralised with sodium hydroxide solution. The Fehling's test in (d) above was repeated. A brick red colour indicates the presence of combined reducing sugars (Evans, 1996).

Resorcinol or Salivanoff's Test (Standard Test for Ketoses)

To 2ml of each of the water extract was added a few crystals of resorcinol and an equal volume of conc. Hydrochloric acid, then heated over a bunsen burner flame. A rose colour indicates the presence of Ketoses (e.g. Fructose) (Trease and (Evans, 1996).

(f) **Test for Pentoses**

To 2ml of each of the water extract was added an equal volume of hydrochloric acid containing little phloroglucinol and heated over a bunsen flame. A rose colour indicate the presence of pentoses (e.g. xylose) (Evans, 1996).

2.7.2 Test for Tannin

a. Extraction

3g of each of the powdered samples was boiled in 50ml of distilled water for 3 minutes on a hot plate. The mixture was filtered and the resulting filtrate was used to carry out the following tests for tannins.

b Ferric Chloride Test

A portion of each of the water extracts was diluted with distilled water in a ration of 1:4 and a few drops of 10% ferric chloride solution was added. A blue or green colour indicates the presence of tannins (Evans, 1996).

c Lead Subacetate Test

To 1ml of each of the water extract was added 0.25% ferric ammonium citrate solution. To the mixture was added sufficient solid sodium acetate to adjust the solution to P" 8 using "P indicator paper. This was boiled in a water bath and filtered. A coloured precipitate indicates the presence of tannins (Evans, 1996).

d Bromine Water Test

A few drops of bromine water was added to 1ml of each of the water extract. A precipitate indicates the presence of condensed tannins.

e Formaldehyde Test

To a small portion of each of the water extract was added a drop of formaldehyde solution and 3 drops of 10% hydrochloric acid. A precipitate indicates the presence of hydrolysable tannins.

f Test for Chlorogenic Acid

To 1ml of each of the water extract was added two drops of 10% ammonia solution. The mixture was heated over a flame and then exposed to air. A green colour indicates the presence of chlorogenic acid (Evans, 1996)

g Lime-water Test

To a small portion of each of the water extract was added 1ml of lime-water. A precipitate indicates the presence of psuedotannins.

2.7.3 Test for Glycosides

a Test for cyanogenic glycosides

A malls amount of each of the powered samples (leaves, stem, stembarks and seeds) was placed in a test tube and sufficient distilled water was added to cover the samples. A prepared moist sodium picrate paper was suspended in the neck of the tube by means of a cork. The tube was placed in a water bath for one hour. A brick-red colour on the paper indicates the presence of cyanogenic glycosides (Evans, 1996).

b Test for Anthraquinone Derivatives

i. Bontrager's test for Free Anthraquinones

0.5g of the powdered samples (leaves, stem, stembarks and seeds) was taken in separate dry test tubes and 10ml of chloroform was added and shaken for 5mins. The extract was filtered and equal volume of ammonia was added to the filtrate and shaken. A bright pink colour in the upper aqueous layer indicates the presence of free anthraquinones (Evans, 1996).

ii. Bontrager's test for Free and / or Combined Anthraquinones

0.5g of each of the powdered samples (leaves, stem, stembarks and seed) was boiled with 10ml of 10% hydrochloric acid for 2min. the extract was filtered. To the filtrates was added equal volume of chloroform. The tube was inverted a couple of times avoiding vigorous shaking. The solution was transferred into a separating funnel and the two layers allowed to separate. The lower chloroform layer was poured into a clean test tube and 10% ammonia solution was added and shaken. The two layers were again allowed to separate. A bright pink colour in the upper equeous layer indicates the presence of free and / or combined anthraquinones ((Evans, 1996)).

iii. Bontrager's test for Anthraquinone Derivatives in a Reduced Form which are not easily Hydrolysed

0.5g of each of the powdered samples (leaves, stem, stembarks and seeds) was boiled with 10ml of 10% $feCl_3$, and 5ml of 10% HCl for 5min. the mixture was filtered and to the filtrate was added equal volume of chloroform. The layers were allowed to separate in a separating funnel. The chloroform layer was transferred into another tube containing 5ml of 10% ammonia solution. A bright pink colour in the upper aqueous layer indicates the presence of anthraquinones (the C-glycosides) (Evans, 1996).

c. Test for Saponins

i. Froth test

To a small quantity of each of the powdered samples was added 95% ethanol. The mixture was filtered and 2.5ml of the filtrate was added to 10ml of distilled water in a test tube. The test tube was stoppered and shaken vigorously for about 30 sec. then it was allowed to stand for over half an hour. A honey-comb froth is indicative of the presence of saponins (Sofowora, 1993).

ii. Haemolytic test for saponins (steroidal and triterpenoid)

0.2g of the powdered leaves and stem-bark were extracted with 10ml of warm water, filtered and the filtrate retained. 2ml of 1.8% sodium chloride solution was added to two test tubes. The concentration of NaCl in each case is now isotonic with blood serum. Blood was obtained by pricking the thumb at the base of the nail and the blood was drawn into a small pipette. Five drops of the blood was added to each of the test tubes labelled. The tubes were stoppered and inverted gently to mix the contents. Haemolysis in the test tube containing extract indicates presence of saponins (Brain and Turner, 1975).

d. Tests for Cardiac Glycosides

i. Extraction

0.5g of each of the powdered sample was boiled with 10ml of 95% alcohol for 2 min. the resulting mixture was filtered and cooled. The filtrate was diluted with water and three drops of a strong solution of lead sub-acetate was added. This was mixed thoroughly and filtered. The filtrate was divided into two portions. One portion of the filtrate was kept for the test described below. The other portion of the filtrate was extracted with 5ml chloroform in a separating funnel. The lower chloroform layer was divided into two small evaporating dishes and evaporated to dryness.

ii. Keller – killiani test for digitoxose

One of the chloroform residues from above was dissolved in 1ml of glacial acetic acid containing a trace of ferric chloride solution.

This solution was carefully poured on the surface of 1ml sulphuric acid already contained in a test tube to form a separate layer. A reddish-brown colour at the interface of the liquids indicates the presence of digitoxose. (Evans, 1996).

iii Kedde test (for free or combined cardenolide aglycone)

The reserved filtrate (from above) was treated with 1ml of 2% solution of 3, 5-dinitrobenzoic acid in 95% alcohol. The solution was made alkaline with 5% sodium hydroxide. A purple-blue colour indicates the presence of free or combined cardenolide aglycone (Sofowora, 1993).

iv Legal Test (for Cardenolide Aglycone)

The second reserved residue (from di) was dissolved in a few drops of pyridine and a few drops of 2% sodium nitroprusside was added then, a drop of 20% NaOH was added. A deep red colour indicates the presence of a cardenolide aglycone (Sofowora, 1993).

e. Test for Terpenes and Sterols

i. Extraction

5g of each of the powdered samples was extracted by maceration with 50ml of ethyl alcohol (95%), filtered and the filtrate was evaporated to dryness. The residue was dissolved in 10ml of anhydrous chloroform and then filtered. The filtrate was divided into two equal portions and the following test were carried out.

ii. **Liebermann – burchard test**

The first portion of the chloroform solution (from (a) above) was mixed with 1ml of acetic anhydride, followed by the addition of 1ml of conc. Sulphuric acid down to wall of the test tube to form a layer underneath. The formation of a reddish violet colour at the junction of the two liquids and a green colour in the chloroform layer indicates the presence of terpenes (Sofowora, 1993).

iii. Salkowski's test

The second portion of the solution was mixed with 2ml of conc. Sulphuric acid carefully so that the sulphuric acid formed a lower layer. A reddish brown colour at the interface indicates the presence of a steroidal ring (Sofowora, 1993).

f. Test for Flavonoids

i. Extraction

5g of each of the powdered samples was completely detanned with acetone. The residue was extracted in warm water after evaporating the acetone on a water bath. The mixture was filtered and the filtrate was used for the following tests.

ii. Lead acetate test

To 5ml of the detanned water extract was added 10% lead acetate solution. A coloured precipitate indicates the presence of flavonoids.

iii. Sodium hydroxide test

5ml of 10% NaOH was added to an equal volume of the detanned water extract. A yellow solution indicates the presence of flavonoids (Evans, 1996).

iv Ferric chloride test

2ml of the detanned water extract was diluted with distilled water in a ration of 1:4 and a few drops of 10% $FeCl_3$, solution was added. A green or blue solution indicates the presence of phenolic nucleus.

v. Shinoda test

0.5g of each of the powdered samples was extracted in ethanol by boiling in a water bath for 5 min. filtered and cooled. To the filtrate was added four pieces of magnesium fillings followed by few drops of conc. Hydrochloric acid. A pink or red colour indicates the presence of flavonoids (Geissman, 1962).

vii. Amyl alcohol test

3g of each of the powdered samples was macerated in 50ml of 1% HCl and filtered.

4ml of the filtrate was shaken with 5ml of amyl alcohol and the colour produced was observed. Production of a yellow colour indicates the presence of free flavonoid aglycones.

10ml of the filtrate was shaken with 7ml of amyl alcohol and the mixture transferred to a separating funnel. The amyl alcohol layer was discarded and the aqueous layer boiled with 10ml of 10% HCl for 2 mins. The acidic solution was cooled and divided into two portions.

The first portion was shaken with amyl alcohol. Production of a yellow colour indicates the presence of combined flavonoid.

To the second portion magnesium turnings were added and the colour of the solution observed (Abdurahman, 1986; Hilal, 1976); production of a red colour indicates the presence of flavanone and flavonol glycosides.

g. Test for Resins and Balsam

i. Test for resins

15ml of petroleum ether extract was made from 0.1g, of each of the powdered samples (leaves, stem, stem bark and seeds) and filtered into a test tube. An equal volume of copper acetate solution was added and shaken vigorously then allowed to separate. A green colour indicates the presence of resins.

0.5g of each of the powdered samples was dissolved in acetic anhydride and 1 drop of concentrated sulphuric acid was added. A purple or violet colour indicates the presence of resins. (Evans, 1996)

ii. Test for oleo-gum-resins

0.1g of each of the powdered samples was triturated in 0.5g of sand. The mixture was transferred into a test tube containing 3ml of ether and shaken. This was filtered into an evaporating dish and the filtrate allowed to evaporate to a thin film. In the fumecupboard bromine vapour was passed over the residue. A violet colour indicates the presence of oleo-gum-resins.

iii. Test for balsams

2 drops of alcoholic ferric chloride solution was added to 5ml of 90% ethanol extract of each of the powdered samples, (leaves, stem bark) a dark green colour indicates the presence of balsams.

h. Test for alkaloids

i. Extraction

10g of each of the powdered samples was taken in a small beaker and a strong solution of ammonia solution was added in a quantity sufficient to just moisten it and allowed to stand for 10min. after thorough mixing of the contents.

Sufficient quantity of a mixture of chloroform and ethanol solution (1:1) was added just to soak and suspend the powder. The mixture was allowed to stand for 20 minutes with occasional stirring with a rod.

The mixture was filtered through a plug of cotton wool. The marc was washed twice with 2ml of chloroform and the washings were combined with the filtrate. The bulked filtrate was concentrated to dryness over water bath. The residue was cooled and dissolved in 5ml chloroform only.

The chloroform solution was transferred to a small separating funnel and shaken with 3ml of dilute sulphuric acid.

The two layers were allowed to separate, the chloroform lower layer was drained off and discarded. 3ml of chloroform was further added and shaken, drained off and discarded until upper acid layer was colourless.

The acid layer was made completely alkaline with strong ammonia solution (tested with indicator paper). The extraction with 3ml of chloroform extracts were retained and evaporated to dryness (Brain and Turner, 1975).

The residue was dissolved in 3ml of ethanol and the following tests were carried out after neutralising with dilute sulphuric acid.

ii. General test

A set of two test tubes were taken for each of the different drugs samples. To each little amount of the ethanolic solution from above was added drop wise a few drops of the following reagents:-

Mayer's reagent - (Potassium mercuric iodine solution)

Dragendorft's reagent - (Potassium bismuth iodine solution)

Wagner's reagent - (Solution of iodine in potassium iodine)

Hager's reagent - (A saturated solution of picric acid)

The presence of precipitate in at least 3 or all of the above reagents indicates the presence of alkaloids (Evans, 1996).

2.8 Extraction of leaves of *Hymenocardia acida*

The powdered leaves (100g) were macerated petroleum ether (boiling point 60^0 – 80^0C) to yield light petroleum ether extract subsequently referred to as (A). The marc was then macerated with 95% methanol concentrated to yield a syrup residue. The ether portion was evaporated in vacuo to yield a residue subseqeuntly referred to as (B). The remaining aqueous portion (1) was partitioned with 3 portions of butanol (each 100ml), the aqueous portion was evaporated in – vacuo to yield a residue subsequently referred to as (I). The butanol portion was partitioned with 3 portions (each 100ml) of 1% aqueous potassium hydroxide, the butanol portion was

evaporated in vacuo to yield a residue subsequently referred to as (2), while the aqueous potassium hydroxide portion was neutralized with dilute hydrochloric acid and partition with butanol.

The final butanol portion was evaporated in – vacuo to yield a residue subsequently referred to as (3). The different portions (1), (2) and (3) were subjected to various test for the presence of phytochemical constituents (Loan, 1981; Evans). Fractions A, B, C were not used. This was dissolved in aqueous methanol and percolate with diethyl ether (Fig. 10).

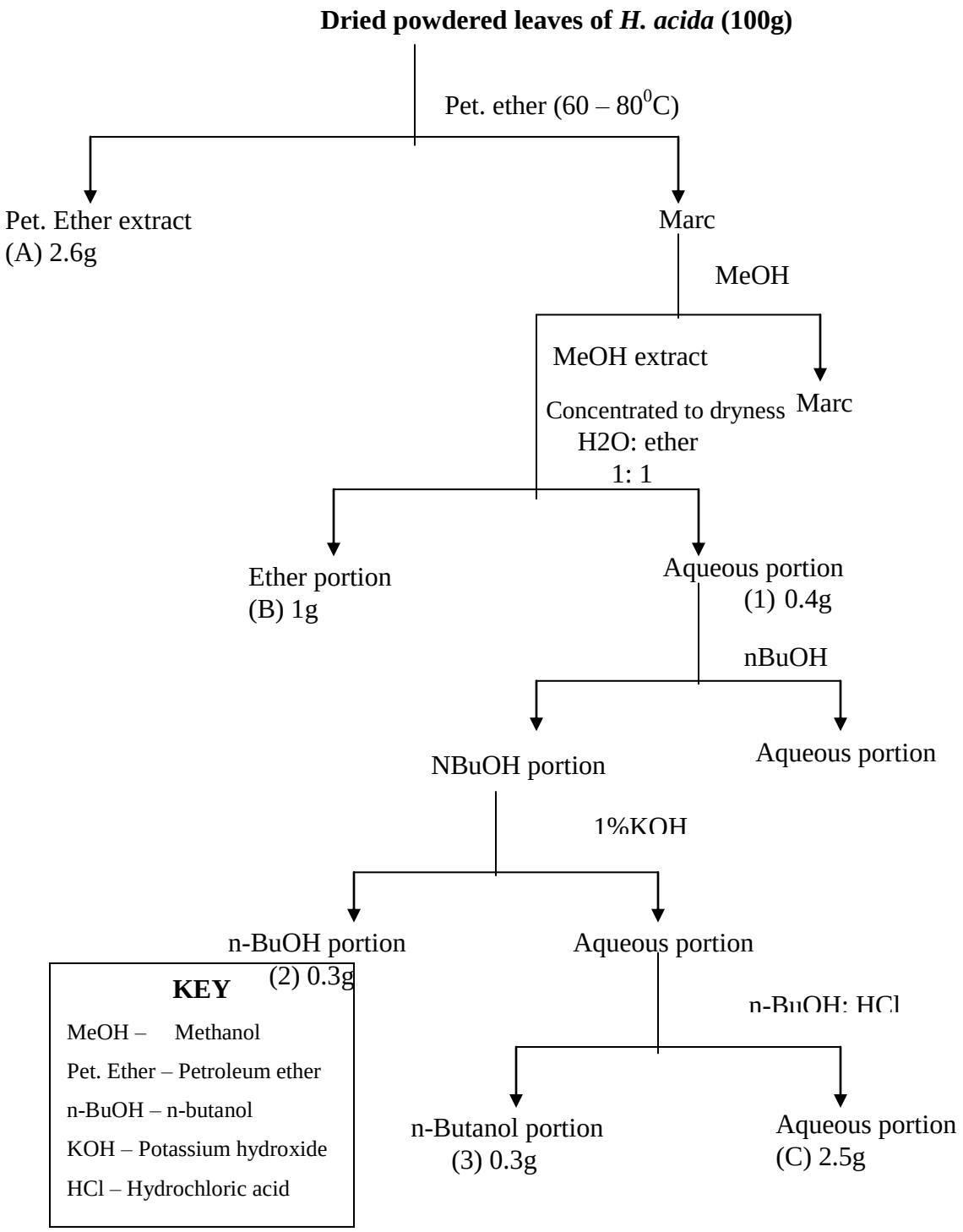


Fig.10: Extraction of the leaves *H. acida*(Won et al, 1980)

2.9 Chromatographic Studies

2.9.1Thin layer chromatographic studies of the alkaloids extracts of the leaves *of Hymenocardia acida*

The identity of the alkaloid extract was investigated using thin layer chromatographic techniques on silica gel $G6F_{254}$ using the following solvent systems

System 1 = solvent system: chloroform – Ethanol (9:1)

System 2 = solvent system: chloroform – methanol (3:1)

Spots were monitored in iodine vapour (as a general reagent), florescence at 254nm and using some specific reagents (Dragendorff,s reagent etc). Identification of spots was also done by determining the Rf values.

2.9.2 Column chromatographic studies of the alkaloidal extract of the leaves of *Hymenocardia acida*

This was separated by column chromatography on silica gel (Touchstone; Dobbins, 1987). 0.5g was packed on a silica gel (60 – 120 mesh) column of 50 x 1.2 cm dimension; the column was eluted using gradient technique from pure petroleum ether (60-80 ^{O}C), various mixtures of petroleum ether and chloroform through pure chloroform. The column was developed at a rate of 1m/min and 10ml fractions were collected. A total of 100 fractions were collected. The progress of Elution was monitored via TLC using the solvent system: Pet: $CHCl_3$ (9:1) and chloroform (100%). Similar fractions were pooled together based on their TLC profiles.

2.10 Evaluation of biological activity of extracts of the leaves of *Hymenocardia acida*

2.10.1 Experimental animals

The animal care and handling were conducted in compliance with standard and humane procedures, which are consistent with International Animal Welfare Guidelines (Rollin an Kesel, 1990).

White albino rats (Wister strain, Rattus norvegecus) and BALB/C mice(Mus musculus) bred at the animal house of the department of pharmacology and clinical pharmacy, A. B. U. Zaria were used.

2.10.2 Acute toxicity studies of the extracts of *Hymenocardia acida*

The Lorke method (1993) and Duffus (1980) modified were used for this study. Doses of 500, 1000, 2000, 3000, 4000, 5000 mg/kg body weight(bw) and 8 mice; doses of 10000 , 11000 mg/kg bw and 3 mice were administered orally.

2.10.3 Chronic toxicity studies of powdered leaves and stem-bark of *Hymenocardia acida*

(a) Histopathology

In traditional medicine *Hymenocardia acida* is usually administered over long periods, the aim of the experiment is to monitor the effect of the plants in animal tissues and organs.

Wistar rats aged 4 – 6 weeks were used and were grouped in to control and treated groups. The control groups were given un-amended diet, this consisted of ECWA grower mash, powdered, cassava as binder and sufficient quantity of warm water. The treated groups were given amended diet containing 25% and 50% powdered plant material (leaves or stem bark).

The experimental animals were grouped into two groups (6 rats per group). One groups was fed with 25% w/w amended diet for 90 days, the other was control group.

In this case, pre and post-treatment water-intake, feed-intake, and body weights of each rat were recorded on a daily basis. At the end of the experimental period, the rats were sacrificed; their various organ weights were taken and recorded. The organs taken for necropsy were liver, kidney, testis, intestine, spleen, stomach, heart, brain, lung and skin for histopathological studies. They were fixed in 10% buffered neutral formalin for at least 48 hours.

Various organs were taken from the dead and euthenaised animals at post-mortem from each of the experimental groups mentioned above. They were fixed to preserve the morphological and microscopical features of the organs. The tissues were fixed in 10% buffered neutral formalin for at least 48 hours before processing. This was to maintain the tissues as it was at ante-mortem.

The tissues were labelled and dehydrated (to remove water from the tissues) in graded series of alcohol in ascending order 70%, 80% 95% and 100% (Absolute) alcohol.

The tissues were cleared in xylene, which is a clearing agent and they were impregnated in paraffin wax. The tissues were separately embedded in paraffin wax for sectioning with rotary microtome and microtome knife. The tissue sectioned at six (6µ) thick and were mounted on clean and grease free slip and died in an oven The stained slides were examined microscopically at X40 objectives and results were recorded. This was repeated with another set of 6 animals per groups fed with 50% w/w amended diet for 65 days and a control group.

(b) Haemotology

This experiment was aimed at evaluating the effects of the plant on the blood parameters with the view of substantiating claims of the usefulness of the plant in anemia in Hausa traditional medicine. The parameters determined were full blood count or differential count for complete blood count (CBC), the packed cell volume (PCV), Mean haemoglobin concentration (MHC), total red blood cell count (RBC count), mean coposcular volume (MCV), total white blood cells count (WBC count), differential leucocyte count, and platelet count. These blood parameters have implications in conditions related to anemia. Wistar rats were fed with amended diets containing 25% and 50% of the leaves and stem bark of *Hymenocardia acida*. After 65 days, the animals were euthanized and the blood parameters were evaluated using standard methods Akinloye and Olorede (2004), the results were compared with animals fed on un-amended diets (without the powdered plant)

2.10 .4 Analgesic activity studies of extracts of *Hymenocardia acida* studies

This experiment is aimed at evaluating the claim of usefulness of the plant in pain relief in Hausa traditional medicine. The analgesic effect of extract of *Hymenocardia acida* was evaluated by the acetic acid induced writhing test.

Mice were grouped into 10 (each of 5 animals). Group 1 were given acetic acid (0.7% v/v in saline) intraperitoneally *ip;* positive controls group 2, 3 were given novalgin (dipyrone 50mg and 100mg) 30mins before acetic acid injections, treated Group 4, 5, 6, 7, 8, 9 were given *H. acida* extracts (sample 1, 2, 3 as indicated in figure at doses of 50mg, 100mg 30mins before acetic acid injections. Negative control group 10 controls were given only 0.1ml normal saline ip.

The number of abdominal constriction produced in each group for the succeeding 5mins was counted and compared to the response in the negative control group. Calculated as the percentage inhibition of abdominal constriction:

CHAPTER THREE

3.0 RESULTS

3.1 PLANT COLLECTION AND IDENTIFICATION

The identity of the plant was further confirmed by comparison of the prepared herbarium specimen with those available at the Department of Biological Sciences Ahmadu Bello University Zaria and the herbarium specimen number (7108) was given. Plates 1-5 show the different illustrations of the plant *H. acida*.

3.2 Apparatus

The apparatus used were obtained from known manufacturers.

3.3 Reagents

The reagents used were obtained from known manufacturers.

Plate 1: The plant *Hymenocardia acida* growing in its Natural habitat


Flowers

Plate 2: Branches and flowers of the plant *Hymenocardia acida*

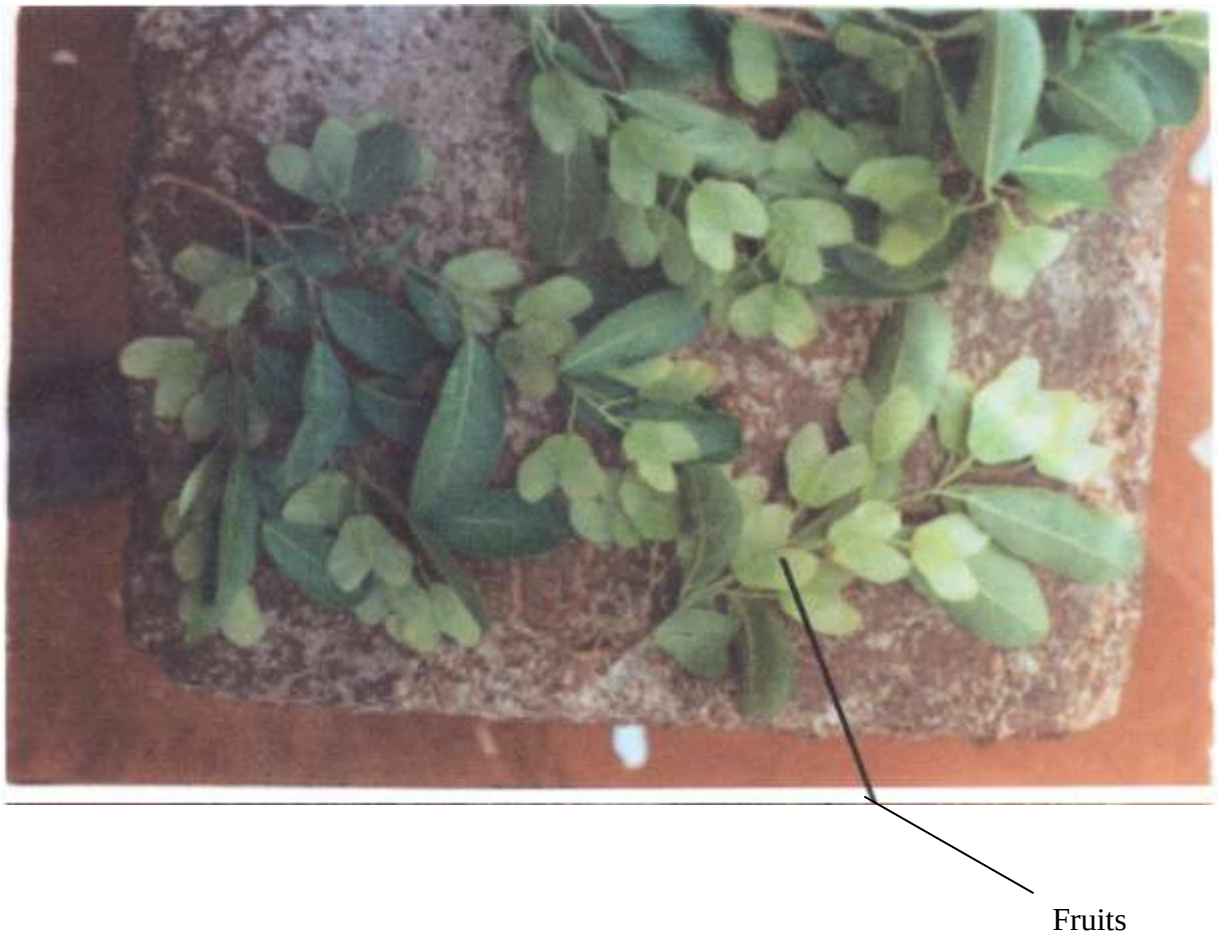


Plate 3: Fruiting branches of the plant *Hymenocardia acida*

Plate 4: Fruits and seeds of the plant *Hymenocardia acida*

Plate 5: Stem bark of the plant *Hymenocardia acida* (showing the characteristic reddish brown colour of the stem)

3.4 Macroscopical examination of the stem bark and leaves of *Hymenacardia acida*

Macroscopical studies revealed characteristic features in the bark and leaves of *H. acida* .

a. Stem bark

The characteristic macroscopical features of the Stem bark was described using standard descriptive terms as follows:

Size	-	Length 1.4 – 3.6cm
		Width 1.5 – 2.8cm
		Thickness 0.05 – 0.1cm
Shape	-	Channelled
Colour	-	Dark brown (inner surface)
		Orange brown (outer surface)
Fracture	-	Smooth and sharp
Surface	-	Soft (fresh)
		Rough (dried)
Odour	-	Slight odour
Taste	-	Pungent, bitter and mucilageneous

b. **Leaves**

The characteristic macroscopical features of the whole leaves was described using standard descriptive terms as follows:

Duration	-	Evergreen
Arrangement	-	Alternate, simple
Petiole	-	Slender, 5cm – 7cm
Composition	-	Simple
Shape	-	Elliptic
Venation	-	Reticulate
Margin	-	Entire
Apex	-	Acute
Base	-	Cordate
Surface	-	Glaborous with naked eye
Size	-	Length - 6.7cm
		Width - 3.4cms
		Thickness - 0.05cm
Colour	-	Dark green
Odour	-	None

Taste -slightly bitter

3.5 Microscopical examination of the stem bark and leaves of *Hymenacardia acida*

a. Stem-bark

The microscopical examinations of the powdered stem-bark revealed the following characteristic features; circular and rectangular secretory cells which are very few in number which also contains oils. Frequent polygonal sclereids, frequent non-articulated laticifers, rectangular and elongated with straight anticlinal walls cork cells, tapering phloem fibres with narrow lumen, uniseriate, multicellular, unicellular trichomes which are moderate in number, polygonal parenchyma, simple and compound starch grains which are oval in shape. Few sieve tubes and numerous calcium oxalate crystals which are needle-like, prisms and rosettes in shapes. Figure 11 shows the characteristic features of the powdered stem bark of *H. acida*.

The various microscopical features of the powdered stem-bark are summarised as follows:

Sensory characters

Colour - Brown

Odour - Slight odour

Taste - Pungent,

1. Secretory cells

Type - Oil cells and tannins

Shape - Circular and rectangular

Size - 1µ – 8µ

Frequency - Very few

2. Scleroids

Shape - Polygonal

Size - 62.5µ - 100µ

	Frequency	**-**	**Frequent**
3.	**Laticifers**		
	Types	**-**	**Non articulated and branched**
	Frequency	**-**	**Frequent**
	Size	**-**	**1.3µ - 7.5µ**
4.	**Cork cells**		
	Shape	**-**	**Rectangular and elongated**
	Anticlinal walls	**-**	**Straight**
	Thickening	**-**	**Present**
5.	**Phloem fibres**		
	Size	**-**	**Length 862.5µ**
			Width 32.5µ
	Frequency	**-**	**Few**
	Apex	**-**	**Tapering**
	Lumen	**-**	**Narrow**
6.	**Trichomes**		
	Type	**-**	**Uniseriate, Multicellular, Unicellular**
	Frequency	**-**	**Moderate**
	Size	**-**	**length - 45µ - 125µ**
			Width - 10µ - 12.5µ
7.	**Parenchyma cells**		
	Shape	**-**	**Polygonal**
	Size	**-**	**37.5µ - 62.5µ**

Frequency - Numerous

The transverse section of the stem-bark also gives a typical example of a dicotyledoneous plant with clearly visible medullary rays. Almost all the cells in the xylem area are liguified. Covering trichones are also abundantly present on the stem-bark of the plant (Fig. 12).

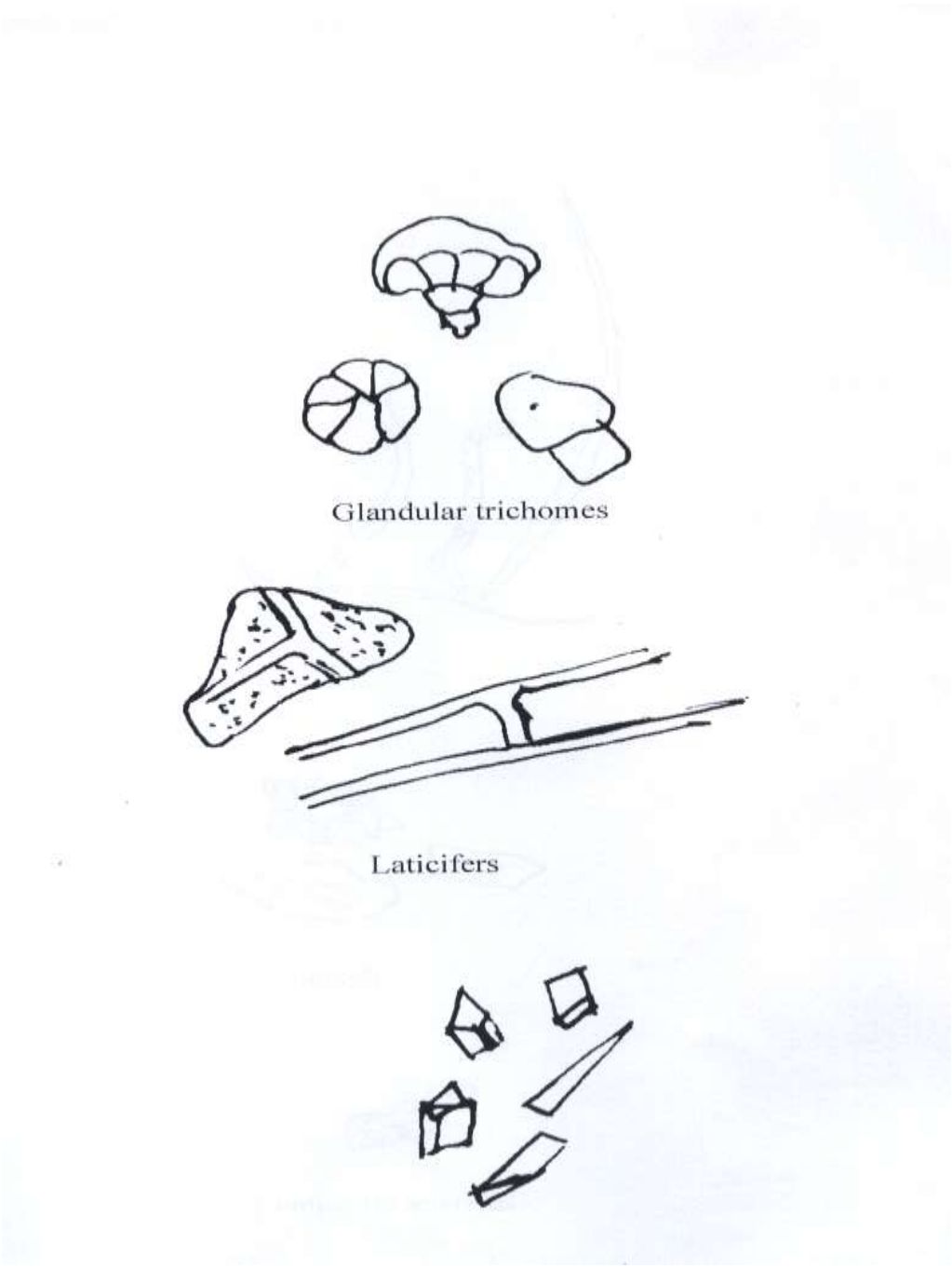
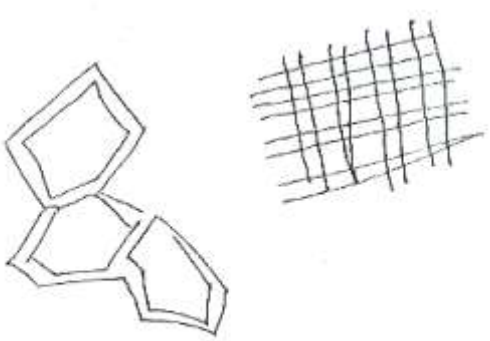
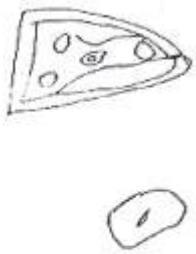


Figure 11: Microscopical features of the powdered stem-bark of *H. acida*



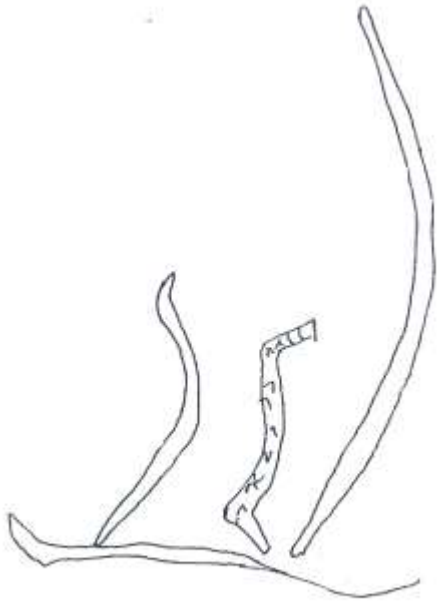
Cork cells



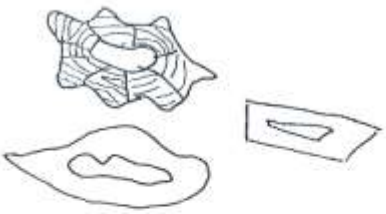
Secretory cells

Sieve tubes

Figure 11 contn



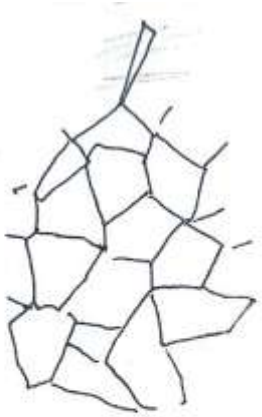
Fibres



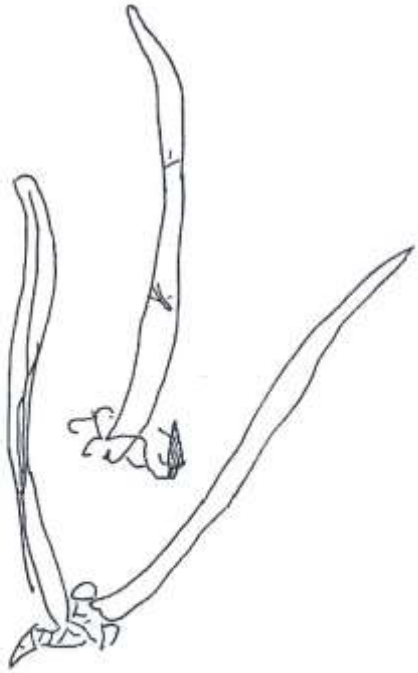
Selereids

Compound selereids

Fig 11 contn



Parenchyma cells



Covering trichomes

Fig 11 contn

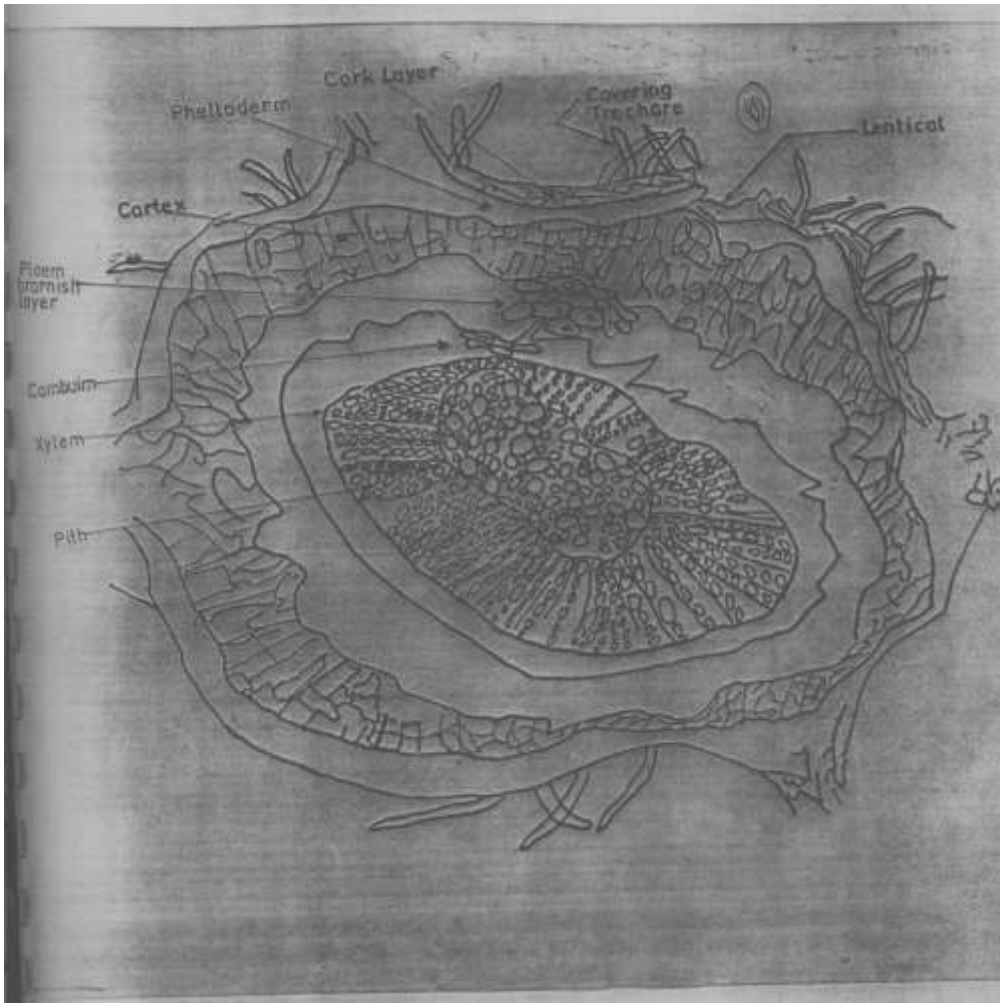


Figure 12: Transverse section of the stem-bark of *H. acida*

b. Leaves

Microscopical examination of the leaves revealed characteristic features of identification. The microscopical features of the powdered leaves sample were as follows Epidermis with stomata, spongy mesophyll, trichomes, laticifers, sclereids, phloem fibres, calcium oxalate crystals, starch grains and secretory cells. These features are shown in figure 13.

The description of the features are as follows:

1. Stomata

Type - Actinocytic (moderate), paracytic (few)

Size - 12.5µ - 20µ

Frequency - Numerous

2. Phloem fibres

Size - Width - 7.8µ

Length - 52.5µ

Frequency - Few

Apex - Tapering

3. Calcium oxalate crystals

Type - Prism, rosette, needle like

Size - (i) Prism

Length - 17.5µ - 40µ

Width - 15µ - 35µ

Frequency - Numerous

(ii) Needle like

Length - 27.5µ - 37.5µ

Width - 6.25µ - 28µ

Frequency - Numerous

(iii) Rosette

Length - 17.5µ - 20µ

Frequency - Scanty

4. Secretory cells

Type - Tannins, oils

Shape - Round

Size - 10µ - 20µ

Frequency - Few

5. Trichomes

Type - Unicellular

Size - Length - 25µ - 52.5µ

Width - 2.5µ - 3.75µ

Frequency - Few

6. Starch grains

Shape - Oval

Size - 2.5µ - 10µ

Aggregation - Mostly single, few appear in clusters

Frequency - Few

7. Parenchyma cells

Type	**-**	**Polygonal**
Size	**-**	**37.5µ - 40µ**
Frequency	**-**	**Numerous**

8. Laticifers

Types	**-**	**Non-articulated and branched**
Frequency	**-**	**Numerous**

9. Cork cells

Shape	**-**	**Rectangular and elongated**
Anticlinal walls	**-**	**Straight**
Thickening	**-**	**Present**

c Anatonomical sections of the leaves and stem-bark of *H. acida*

The anatomical sections revealed the transverse section of the leaves as dorsiventral leaves with the palisade cells below the upper epidermis only. (Fig.14). The transverse section through the midribs is given on Fig. 15.

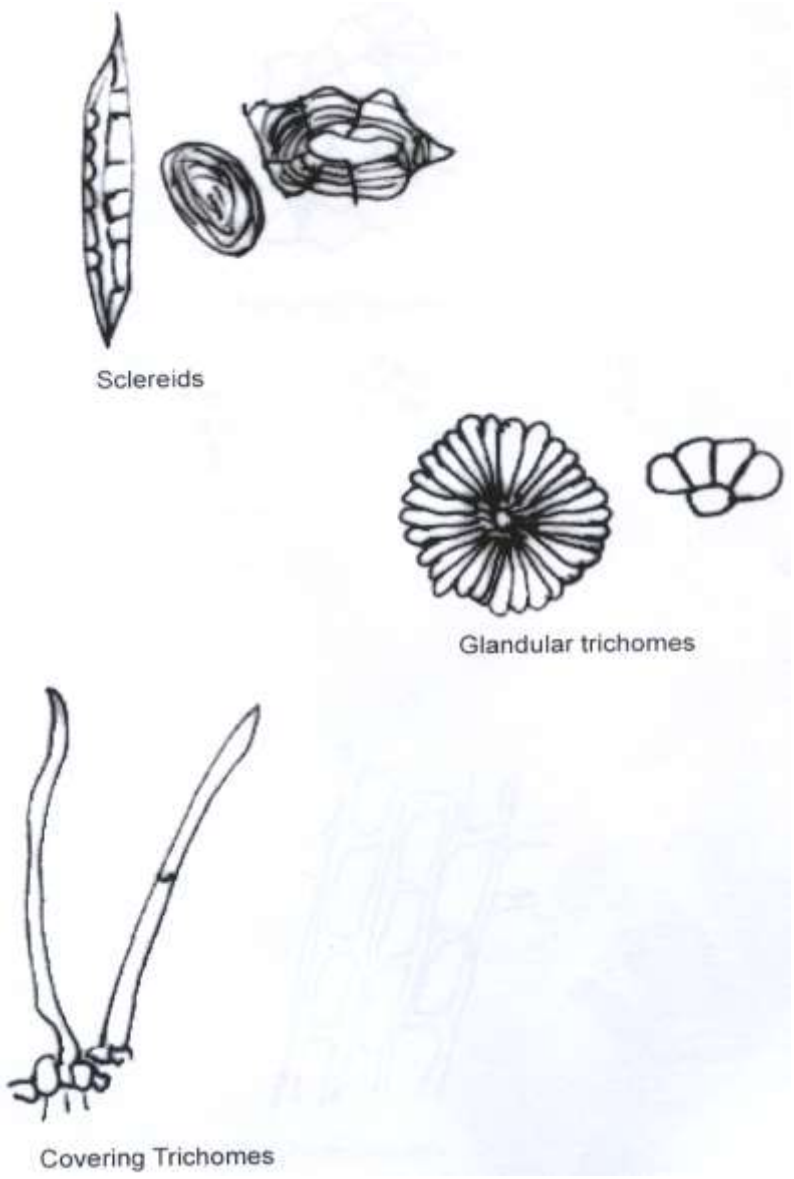
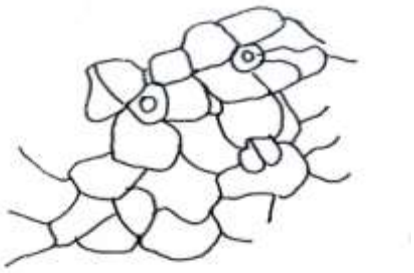
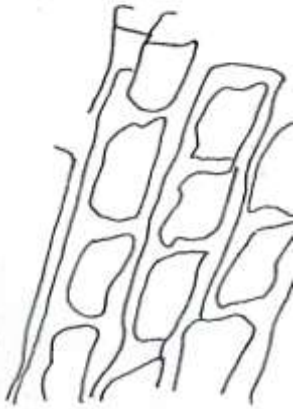


Figure 13: Microscopical features of the powdered leaves of *H. acida*



Animocytic stomata

Calcium oxalate crystals



Parenchyma cells

Fig 13 : contn

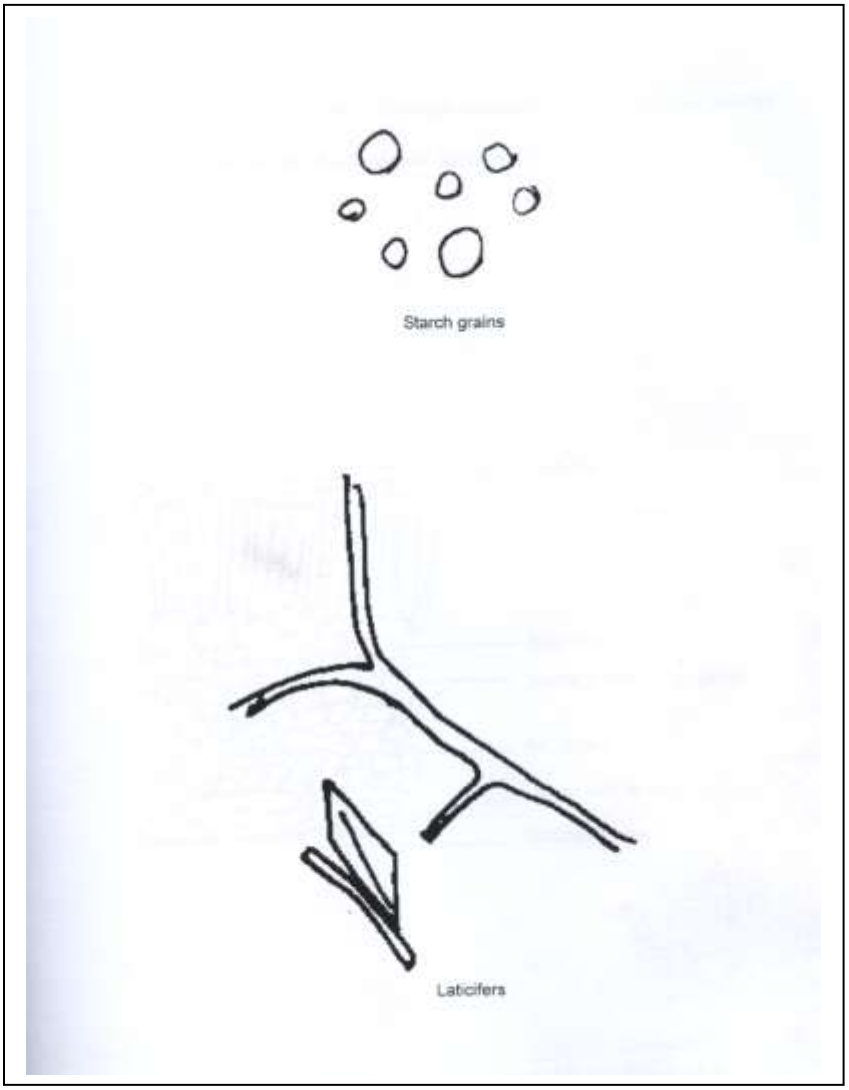


Fig 13 contn

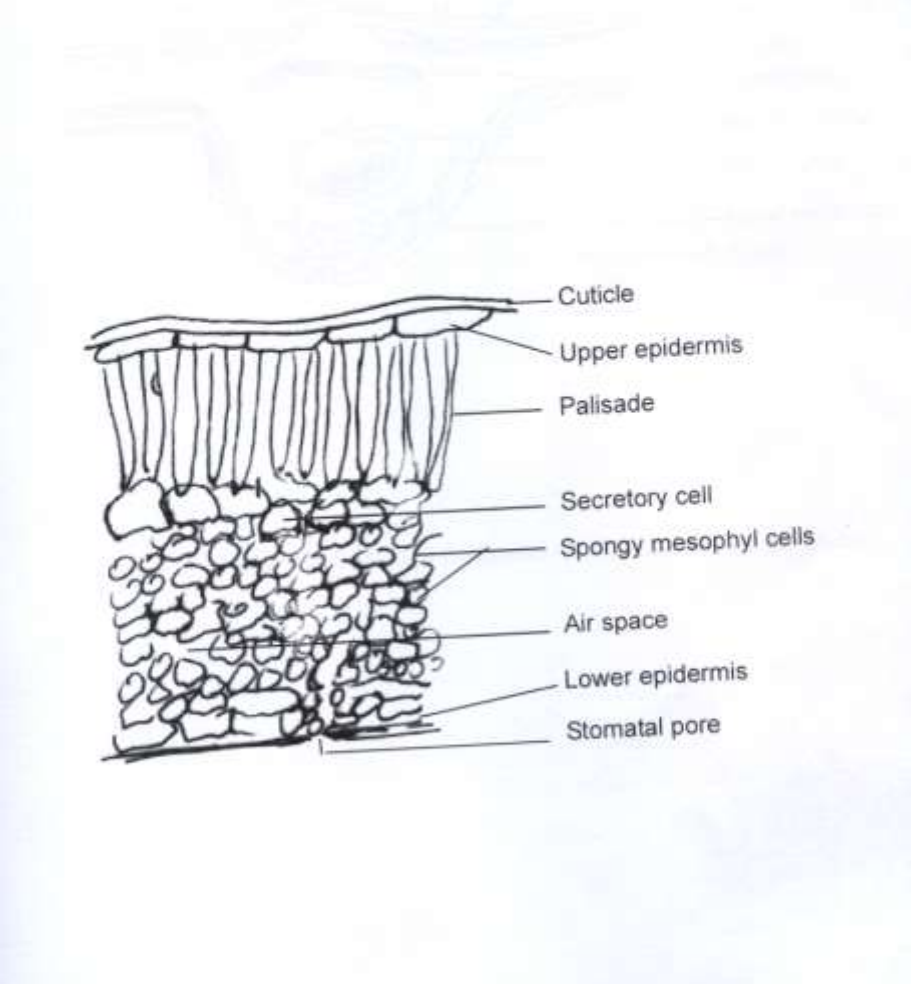


Figure 14: Transverse section of the leaves of *H. Acida* through the lamina

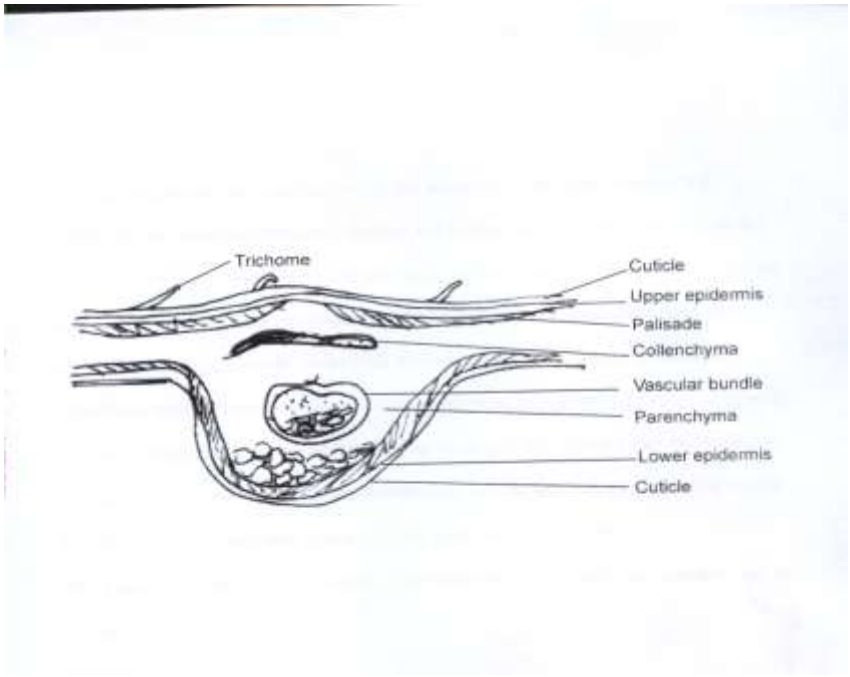


Figure 15: Transverse Section of the Leaves Of *H. Acida* through the Midrib

e Chemomicroscopical examination of the stem-bark and leaves of *H. acida*

i Test for starch:

When N/50 iodine solution was added a blue-black colour was observed indicating the presence of starch. The leaf had the largest amount of starch than the stem-bark.

ii **Test for cellulose**

A bluish colour was observed with N/50 iodine solution followed by the addition of 66% w/v H_2SO_4 on both the stem bark and leaf powder. This indicates the presence of celluloses

Laticifers, secretory cells, parenchyma cells, cell wall and sieve tubes were all stained blue-black.

iii **Test for lignin**

A red colour was observed indicating the presence of lignin. Sclereids, secretory cells, laticifers, parenchyma cell and fibres were found to be lignified when mounted with phloroglucinol and concentrated acid.

iv **Test for tannins**

Tannin was found to be present in both the stem-bark and leaves powder, because a greenish black and green colour was confirmed when mounted in ferric chloride solution.

v **Test for oil**

Red colouration on some parenchyma cells and some secretory cells was observed with Sudan IV. This indicates the presence of oils or lipids.

vi **Test for mucilages**

Mucilage was found to be present in the leaves powder but absent in the stem-bark powder when mounted in Ruthenium Red solution, pink colour was observed.

vii **Test for calcium oxalate crystals**

The bright structures observed disappeared on the addition of 80% H_2S0_4. This confirms the presence of calcium oxalate crystals.

The result of the chemo-microspical tests is given in table 1.

Table 1: Chemomicroscopical results

Constituents	Leaf	Stem-bark
Starch	++	+
Cellulose	+	+
Lignins	+	++
Tannins	+	++
Oils	+	++
Mucilages	+	-
Calcium oxalate crystals	++	+

(+) - present
(-) - absent

Table 3: Quantitative values for powdered leaves and stem bark of *H .acida.*

Evaluation parameters(% w/w)	Stem-bark % w/w	Leaves % w/w
Moisture content	8.0	20.5
Ash value	11.5	4.8
Acid – insoluble ash value	7.5	1.5
Alcohol soluble extractive value	37	34
Water soluble extractive value	44	42

3.6 Quantitative microscopical examination of the leaf of *Hymenocardia acida*

The quantitative microscopical values of the leaf can provide numerical data for identification, determination of purity, sources, and detection of adulteration (Dasniyam, 1991). The values in table 3 shows the quantitative microscopical values for the leaves of *H. acida*

Table 3: Quantitative leaf microscopical values for *H. acida*

Parameters	Values	
	Range	Mean
Palisade ratio	37 – 75	52.8
Stomatal number	24 – 31	28
Stomatal index	7.7 – 9.2	8.3
Vein islet number	36 – 40	38.0
Vein termination number	6 – 8	7.25

3.7 Phytochemical screening of powdered leaves, and stem bark of *Hymenocardia acida*

The results obtained from the various phytochemical test are described below:

3.7.1 Test for carbohydrates

a. **Extraction**:

Most carbohydrates are wholly or partially soluble in water, the water extracts was therefore used for conducting the various tests for different classes of the carbohydrates.

b. **Molisch's test**

This is a general test for carbohydrates.

A purple ring at the common surface of the liquids (molish's reagent and water extract) and a dull violet precipitate when the mixture of the liquids was shaken, indicated the presence of carbohydrates. Both stem-bark and leaves gave this positive result.

c. Test for sugars

Fehling's test: Test for free reducing sugars. A brick red precipitate was formed

indicating the presence of free reducing sugars in both stem bark and leaves.

When dilute hydrochloric acid (10%) and dil NaOH plus 5ml Fehling's solution A and B was added to the extract, combined reducing sugars were found to be positive as a brick-red colour was obtained.

No precipitate was obtained with Barfoed's reagent with the leaves extract but a red precipitate was observed with the stem bark extract. This indicates that monosaccharides are absent on the leaves but present in the stem-bark.

Salivanorff's Test

A rose colour was obtained which indicates the presence of Ketoses after the addition of resorcinol crystal plus concentrated Hydrochloric acid to the extract.

No colour change was observed when dil. Hcl with phloroglucinol were added to the extract. This indicates that pentoses are absent.

3.7.2 Test for Tannins

Both stem-bark and leaves extracts responded to different tannin tests as follows:

a. Ferric chloride test

Greenish colour was obtained indicating the presence of tannins.

b. Lead subacetate test

White coloured precipitate was observed indicating the presence of tannins. Tannins are precipitated by heavy metals in this case it is precipitated by lead.

c. **Bromine water test**

A brick red coloured precipitate formed, thus indicating the presence of condense tannins.

d. Lime water test

A white coloured precipitate was formed with both the stem-bark and leaves extract which shows that pseudotannins are present.

f. Formaldehyde test

No precipitate was obtained with any of the samples with this reagent indicating the absence of hydrolysable tannins.

From these results, it might be concluded that *Hymenocardia acida* contains condensed tannins and pseudotannins.

3.7.3 Test for Glycosides

a. Test for cardiac glycosides

Positive results were obtained for both stem-bark and leaves with Keller Killiani test, where the extract plus 2ml glacial acid containing $Fecl_3$ + conc. H_2S0_4.

This test indicates the presence of deoxy sugars. With Kedde test: solution of extract in ethanol + 2% 3, 5 – dinitrobenzoic acid in methanol +5.7% NaoH, a positive result was obtained with the stem-bark (that is an immediate violet colour was obtained, with the leaves a negative result was obtained. This indicates the presence of denolide in the stem-bark and its absence in the leaves.

b. Cyanogenetic glycosides

Sample + moistened picrate paper no colour was observed on the picrate paper for both the stem-bark and leaves. This shows that Gynogenetic glycosides are absent.

3.7.4 Test for Saponins

i. Powdered samples + 95% ethanol then diluted with 10ml distilled water and solution saponin was observed to be present in both the stem-bark and leaves as a honey comb both which lasted for mor e than 30 mins.

ii. **Haemolytic test for saponins gave precipitate with both the stem-bark and the leaves.**

3.7.5 Test for Anthraquinone Derivatives

Bontrager's test

A bright pink colour was observed in the upper layer with the leave extract. This indicates the presence of anthraquinones derivatives. No colour change was observed in the upper layer, which means the test was negative with the stem-bark extract.

Powdered sample plus 10% Hcl and chloroform, a pink colouration was observed with the stem-bark powder, but the test was negative with the powdered leaves. This indicates that free and/or combined anthraquinone glycosides are present in the stem-bark and absent in the leaves.

3.7.6 Test for Terpenes and Sterols

i. **Salkowski's Test:** A reddish brown ring was obtained at interface and this indicates presence of sterols in the two different parts of the plant.

ii. **Lieberman – Burchard Test**: A reddish violet ring and green upper solution was obtained for the lieberman – Burchard Test. This shows t hat Terpenes is present in the samples.

3.7.7 Test for Mucilage

Pink colouration was observed under the microscope with the leaf sample but absent in the stem sample.

3.7.8 Test for Flavonoids

The results for the various test for flavonoids are as follows:

i. Lead acetate test

A buff coloured precipitate was obtained thus, indicating the presence of flavonoids in the two samples.

ii. Sodium hydroxide test

Flavanoids are present as a yellow solution was obtained which becomes colourless on addition of dil Hcl.

iii. A greenish colour was obtained with both stem-bark and leaves extracts, indicating presence of phenolic nucleus.

iv. Shinoda test

The two samples gave positive test (a red colour) which confirmed the presence of flavanoids.

3.7.9 Test for Resins and Balsams

Negative results were obtained for the stem-bark extract but positive result were obtained for the leaves extract.

The general phytochemical screening of *Hymenocardia acida* revealed constituents characteristic to the euphorbiaceae.

Table 4 shows a summary of the phytochemical screening of the stem bark and leaves of *H .acida*

Table 4 :Phytochemical constituents of *H. acida*

Phytochemical Tests	Stem-bark	Leaves
1. Carbohydrate test		
- Molisch's test	+ve	+ ve
- Barfoed's test	- ve	- ve
- Fehling's test for reducing sugars.	+ ve	+ ve
- Fehling's test for combined reducing sugars	+ ve	+ ve
- Ketose test	+ ve	+ ve
- Pentose test	- ve	- ve
2. Tannin test		
- Pd subacetate	+ ve	+ ve
- $Fecl_3$	+ ve	+ ve
- Femaldehyde /Hcl	- ve	+ ve
- Branine H_2O	+ ve	+ ve
- Lime water	+ ve	+ ve
3. Glycosides tests		
- Bontrager's test	- ve	+ ve
- Haemolytic test	+ ve	+ ve
- Keller – killiani test	+ ve	+ ve
- Kedde test	+ ve	- ve
- Froth	+ ve	+ ve
- Cynogenetic glycosides	- ve	- ve
5. Flavonoids		
- Lead acetate test	+ ve	+ ve
- NaOH (20%)	+ ve	+ ve
- Shinoda test	+ ve	+ ve
4. Terpenes / Sterols		
- Salkowski test	+ ve	+ ve
- Labemann-Buchards test	+ ve	+ ve
6. Resins / Balsam		
- Cu-acetate	- ve	+ ve
- Acetic hydride and conc H_2SO_4	- ve	- ve
7. Alkaloids		
- Mayers'	+ ve	+ ve
- Dragendoff's	+ ve	+ ve
- Wagners's	+ ve	+ ve

Key: (+ve) - present ; (-ve) - absent

3.8 Extraction of the leaves of *Hymenocardia acida*

Phytochemical and chromatographic analysis of the extracts obtained from fig. 10 gave the following results (Table 5, 6).

Tables 5: Phytochemical screening of the fractions of *Hymenocardia acida* leaves

Samples	**Mayer's** reagent	**$FeCl_3$** test	**Pb acetate** Test	**Pb subacetate** test	**Haemolysis test**	**Inference**
1	**–**	**+**	**+**	**+**	**+**	**Phenolic compounds** & Saponins
2	**–**	**–**	**–**	**–**	**+**	Saponins
3	**–**	**–**	**–**	**–**	**+**	**Phenolic compounds only**
C	**–**	**+**	**+**	**+**	**–**	

Key

+ present

- absent

The phytochemical test did not indicate the presence of alkaloids but TLC did (Table 6).

Table 6 Thin layer chromatographic analysis of the fractions of the leaves of *H. acida*

Samples	R_f value	Dragendorff's spray reagent	Inference
1	0.10	Orange	Alkaloid (+)
2	0.00	Brown	Alkaloid (-)
	0.10	Yellow	"
	0.19	Yellow	"

3	0.00	Brown	Alkaloid (-)
	0.12	Orange	Alkaloid (+)
	0.29	Yellow	Alkaloid (-)
C	0.00	Brown	Alkaloid (-)
	0.13	Orange	Alkaloid (+)

Key

(+) present Solvent system – Chloroform: methanol ; adsorbent – silica gel

(-) absent

3.9 Chromatography

3.9.1 Thin layer chromatographic studies on the leaves and stem bark of *Hymenocardia acida*

The extract of the leaves of H*ymenocardia acida* showed varying spots with the locating reagents, Table 7 shows the results of spots positive with alkaloidal reagents.

Table 7: Thin layer chromatographic results of alkaloidal extract of *H. acida* leaves.

Extract	**R_f value**	**Day Light**	**UV 254nm 366 mn**	**After spray (dragendorff)**
1*	0.33	Visible	No fluoresce	Pale orange
2*	0.12	Visible	No fluoresce	Pale organe
3*	0.2	Visible	No fluoresce	Brown
4*	0.3	Visible	No fluoresce	Brown
5*	0.38	Visible	No fluoresce	Brown
6*	0.5	Visible	No fluoresce	Orange spot
7*	0.6	Visible	No fluoresce	Orange
8**	0.58	Visible	No fluoresce	Orange spot
9**	0.73	Visible	No fluoresce	Brown

Key:

* System 1 = solvent system: chloroform – methanol (9:1)
** System 2 = solvent system: chloroform – methanol (3:1)

3.9.2 Column chromatographic studies of the leaves of *Hymenocardia acida*

The alkaloid extract of the leaves of *Hymenocardia acida* separated by column chromatography gave the following characteristics (Table 8.)

Table 8: Column chromatographic results of alkaloid extract of *H. acida* leaves.

Solvent No.		Fraction No.	Eluting solvent	No. of spots (Iodine)	Dragendorff's	Rf values
1		1 – 7	pet ether (100%)	-		
2	F_1	7 – 28	Pet ether : $CHCl_3$ (9:1)	2 spots	Orange spots	0.04,0.93
3		29 – 35	Pet ether : $CHCl_3$ (8:2)	3 spots	''	0.04,0.12,0.96
4	F_2	36 – 47	Pet ether : $CHCl_3$ (7:3)	3 spots	"	,,
5		48 – 53	Pet ether : $CHCl_3$ (6:4)	3 spots	"	,,
6	F_2	54 – 62	Pet ether : $CHCl_3$ (2:8)	3 spots	"	0.27 0.42
7		63 – 69	$CHCl_3$ (100%)	3 spots	"	0.96 ,,
8	F_4	70 – 75	$CHCl_3$ – MeOH (9:1)	3 spots	"	0.42,0.61.0.96
9	F_5	76 – 80	$CHCl_3$ – meoH (7:3)	1 spot	"	0.95

Key

$CHCl_3$ - Chloroform

Pet ether - Petroleum ether

MeOH - Methanol

Solvent system chloroform: Methanol (9:1)

3.10 Evaluation of biological activity

3.10.1 Acute toxicity studies of the powdered leaves and stem bark of *Hymenocardia acida*

The water extract of H. acida administered orally was found to be safe. There were no death or any sign of toxicity observed within 24 hrs of the doses administered to the mice; even at doses higher than 5000mg/kg ,ie, 10000 mg/kg, and 11,000 mg/kg (Appendix B)

The LD_{50} values greater than 5,000 mg/kg are of no practical interest (Locke, 1983).

3.10.2 Chronic toxicity studies of powdered leaves and stem bark of *Hymenocardia acida*

a) **Histopathology**

The plant is extensively used in Hausa traditional medicine. However, over the experimental period of 90 days, the rats fed with 25% w/w amended feed did not cause any observable tissue damage in the kidneys, brains, livers, hearts, lungs, intestines and spleens in either the leaves or the stem-bark of *H. acida*.

The liver of the control rat had no observable microscopic lesion(plate 6). The rats that were fed with 50% w/w responded differently. The rats that were fed with powdered leaves of the amended feed had focal areas of necrosis of the hepatic cells in the liver (plate 7).

The goblets cells of the intestine were actively producing mucin (plate 8). The spleen had lymphocytes proliferation, active haemopoietic response, haemosiderosis and haemosiderin ladened macrophages (plate 9).

There was no observable microscopic tissue damage in the kidney, heart, brain, lungs and pancreas.

Generally the results have shown that prolonged treatment (chronic) with either the leaves or stem-bark water extracts of *H. acida* may induce hepatotoxicity. This prolonged use as is done in traditional medicine is not recommended and if it has to be done, then it must be with extra care.

The plant has a possible beneficial effect of stimulating haemopoiesis and lymphocytes proliferation which could help in ameliorating anaemic conditions. It may play a role in activating the immune system in the course of prolonged treatment.

The changes in body weight feed and water intake are given on Fig.16,17,18;19 Appendx D, E.

b. Haematology

Also, analyses of the blood parameters (haemotology) showed very significant increase with the leaves of *H. acida* on the packed cell volume (PCV) and Haemoglobin (Appendix f (1, 2). This showed that the plant could be used in the treatment of anaemia. There was no significant change with leaves on TP, RBC, WBC, Neutrophils and lymphocyes Appendix F (5, 7, 9, 11 & 13).

A significant increased of the red cells (RBC) was also observed with the stem-bark (Appendix F (8)), this further proves the plant can be used in correcting

anaemia. Analysis of the stem-bark on the remaining blood parameters, PCV, Hb, TP, WBC, Neutrophils and lymphocytes was not significant (Appendix F(2, 4,6,10, 12,&14).

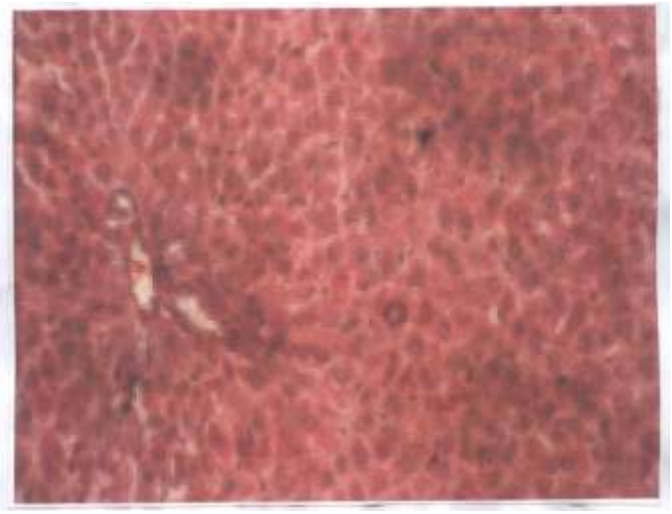


Plate 6: A section of untreated liver (control)

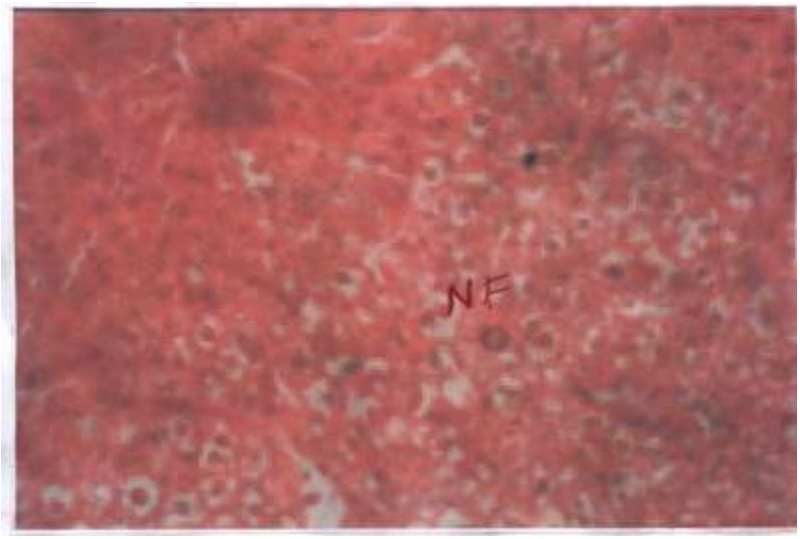


Plate 7: A section of liver taken from a rat treated with 50% of the amended feed for 65 days. Note necrosis of hepatic cells and fatty degeneration (NF). H & E stain (x 400)

Plate 8: A section of liver taken from a rat treated with 50% of the amended feed for 65 days. Note the Goblet cells (G). H & E stain (x 400)

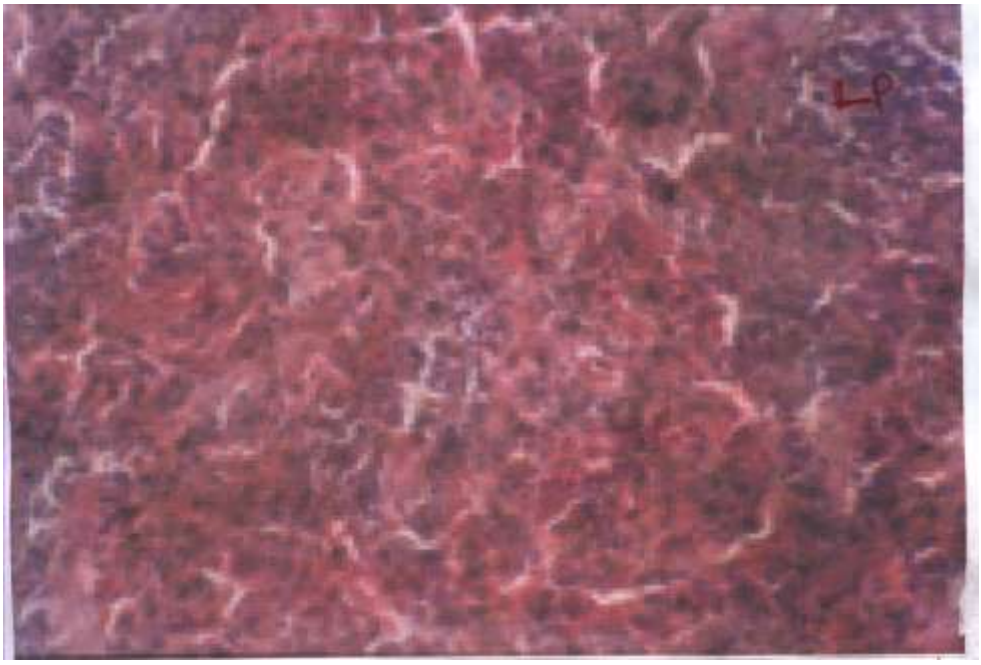


Plate 9: A section from the spleen of a rat that was treated with 50% of the amended feed for 65 days. Note the area of Lymphocyte Proliferation (LP). H & E stain (x 400)

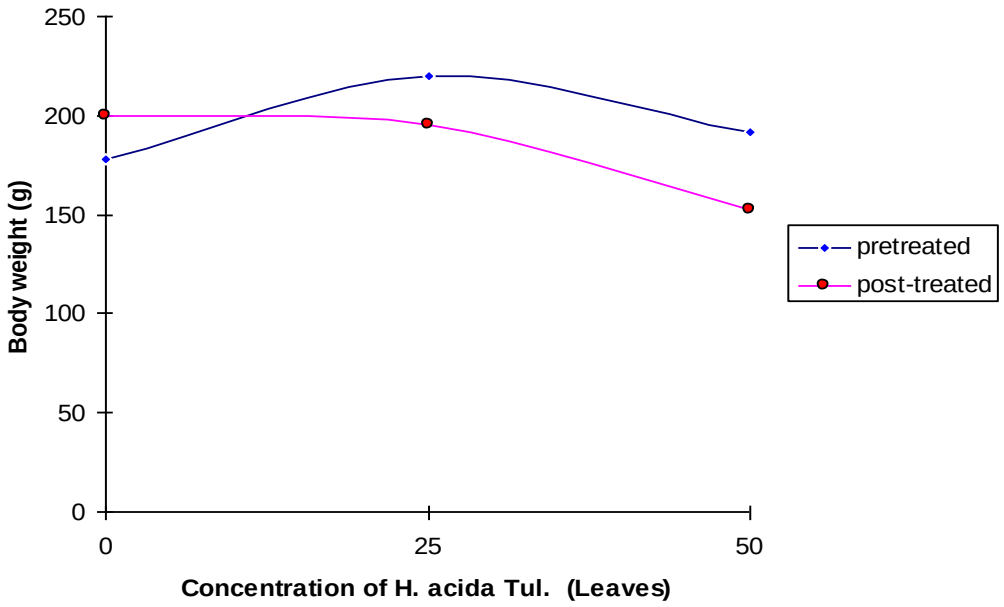


Figure 16: The relationship between pre and post treated mean values of body weight in rats fed with amended diets of *H. acida* leaves

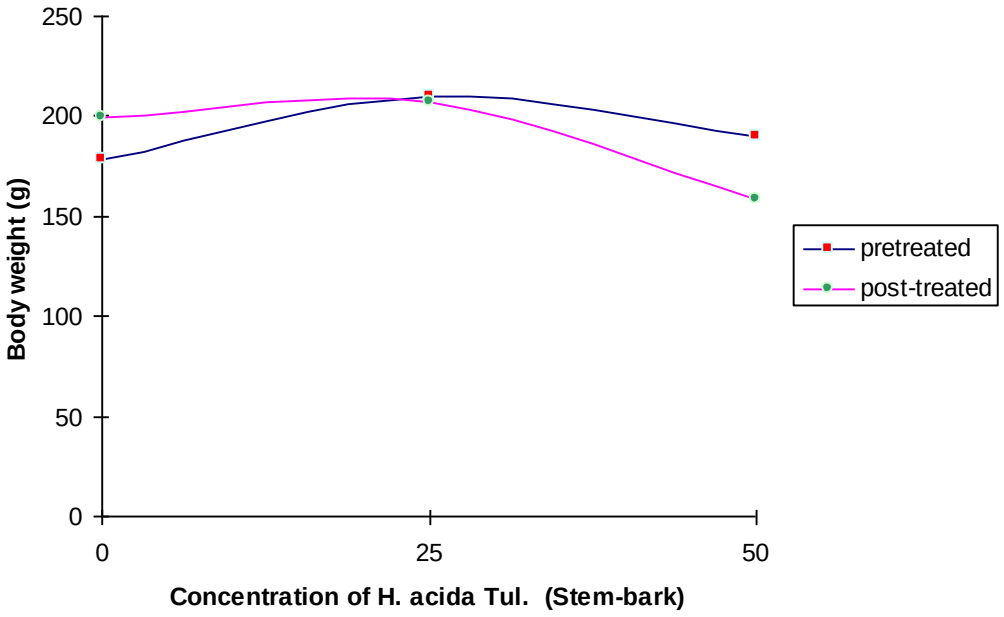


Figure 17: The relationship between pre and post treated mean values of body weight in rats fed with amended diets of *H. acida* stem bark

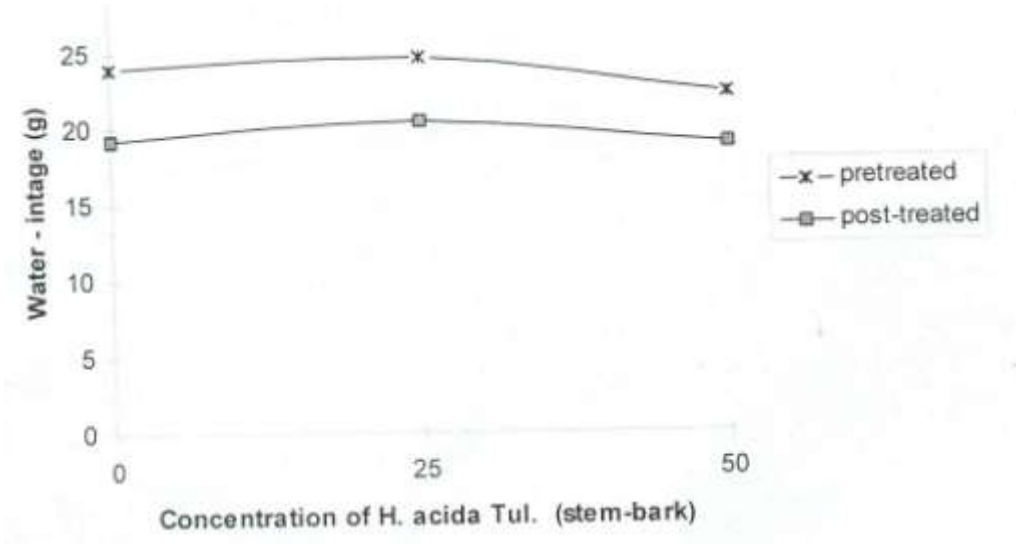
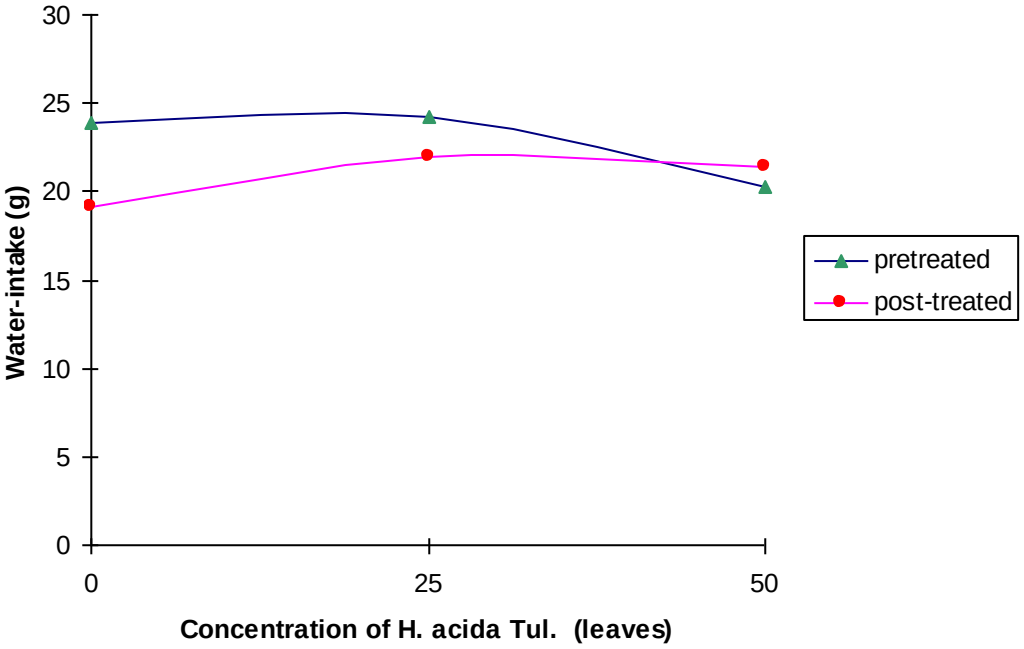


Figure 18 The relationship between pre and post treatment mean values of water-intake in rats fed with amended diets of *H. acida* Stem-bark



(a)

Figure19: The relationship between pre and post treatment mean values of Water-intake in rats fed with amended diets of *H. acida* leaves

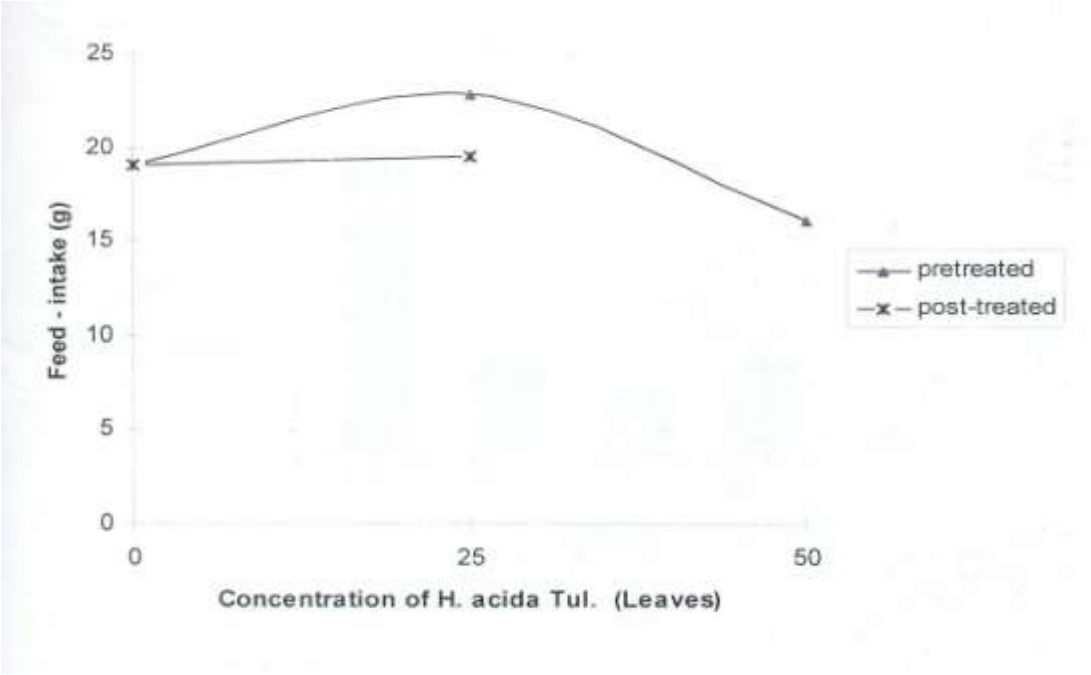


Figure20: The relationship between pre and post treatment mean values of feed-intake in rats fed with amended diets of *H. acida* Leaves

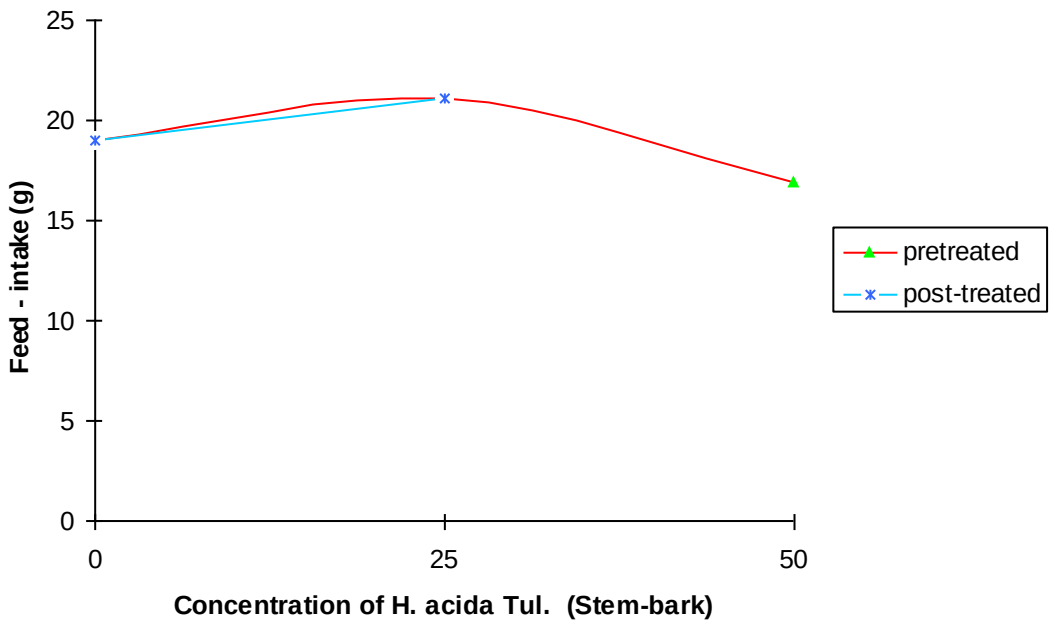


Figure 21: The relationship between pre and post treatment mean values of feed-intake in rats fed with amended diets of *H. acida* stem bark

3.10.3 Evaluation of Analgesic activity of extracts of the leaves of *Hymenocardia acida*

The result showed that the percentage inhibition of abdominal constriction with sample 1, 2 and 3 was significant (at P<0.05 using one way anova). This proves that these fractions containing phenolic compounds, saponins and alkaloids(tables 5, 6) have analgesic properties (Fig.22,23). The saponins and alkaloids, S3 were found to be the most active; followed by phenolic compounds with saponins and alkaloids, S1; then saponins only, S2.

3.11 General Discussion

Macroscopically, the characteristic features of the leaves and stem-bark of this plant observed (plates 1, 2, 3, 4 and 5) were in conformity with the documented characteristic features of the leaves and stem-bark of some members of the family Euphorbiaceae (Eggling 1951).Microscopic, features observed as fibres, sclereids, cork cells, covering trichomes, calcium oxalate crystals, laticifers, glarindular trichomes and animocytic stomata can serve for identification of the plant, the glandular trichoms are peculiar to the plant.

Also the presence of vascular structures when observed transversely indicated the most common vascular structures among dicotyledons occurring in many primitives as well as advanced families as Euphorbiaceae, Rosacae, Compositae and a lot of others are multicellular type (Fig 12, 14, 15).

The phytochemical tests carried out on the stem-bark and leaves of the plant (*Hymenocardia acia*) revealed the presence of alkaloid, saponins, flavonoids, tannins,

cardiac glycosides, anthraquinones and carbohydrates (Table 4,5 and6) . The chemical constituents are responsible for the pharmacological actions of the plants parts. The chromatographic results indicated the presence of alkaloids phenolic compounds and saponins, which are in conformity with both chemonicroscopical and phytochemical test carried out.

The result showed no death or any sign of toxicity with the mice for acute toxicity studies and the rats fed with 25% w/w of *H. cida*. But at higher dose 50% w/w there was evidence of hapatoxicity on the liver tissues.

This further confirms the safety of the drugs at lower doses.

The body weights, water intake and feed intake increased with lower concentration of *H. acida* Tul. 25% w/w (stem bark and leaves) in their feed, but decreased with higher concentration. This showed that the plant proved to be an appetizer and nutritive plant at a lower concentration 25% w/w/ but can be toxic at a higher concentration (50% w/w).

It was observed that the body weight across the pretreated and treated groups with leaves were found to increase significantly. [Appendix D and F] This showed that the plant leaves can be used as a nutritive plant. It was observed that there was no significant change in the pretreated rats with stem-bark [Appendix D] but there was a significant increase in the post-treated rats with stem-bark [Appendix D ($1a_2$)].

Samples 1, 2, 3 showed analgesic activity. These compounds, alkaloids flavonoids and saponins are known to possess analgesic activity (Evans, 1996). The activity was found to dose dependent and significant at $P < 0.05$.

Also, analyses of the blood parameters (haematology) showed very significant increase with the leaves of *H. acida* on the packed cell volume (PCV) and Haemoglobin (Appendix F (1 & 3)]. This showed that the plant can be used in the treatment of anaemia. There was no significant change with the leaves on TP, RBC, WBC, Neutrophils and lymphocytes [Appendix F (5, 7, 9, 11, & 13)].

A significant increased of the red blood cells (RBC) was also observed with the stem-bark [Appendix F (8)], this further proves that the plant can be used in correcting anaemia. Analysis of the stem-bark on the remaining blood parameters, PCV, Hb, TP, WBC, Neutrophils and lymphocytes was not significant (Appendix F (2, 4, 6, 10, 12, & 14)].

The data analyses obtained from analgesic activity indicated that S2 (Saponin extract) showed a very significant reduction in pain [Appendix G (1a, & 2a)], [Appendix H (1a, 1b, 2a, 2b)]. This showed that this extracts has analgesic effect.

Comparison of analgin at different concentrations showed no significant effect. [Appendix H (1b & 2b, 3b)]. This indicated that the analgesic properties of these samples is not as effective as analgin.

3.11 Conclusions

The pharmacognostic values can be used in the identification, differentiation and preparation of a monograph on the plant. The analgesic activity gives justifications for the use of the plant in traditional medicine. Effect on the blood

cells may suggests usefulness of the plant in sickle cell anaemia. Prolonged usage of the plant may be deleterious to the liver , thus overuse should be discouraged.

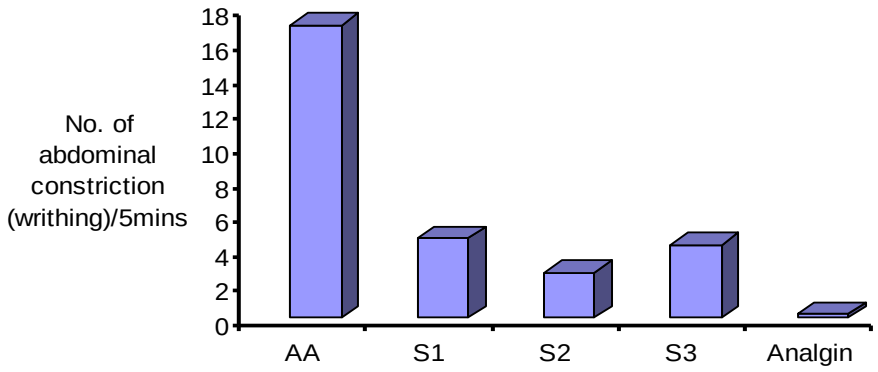


Figure 22: Analgesic activity of 100mg/kg of leaves fractions of *H. acida*

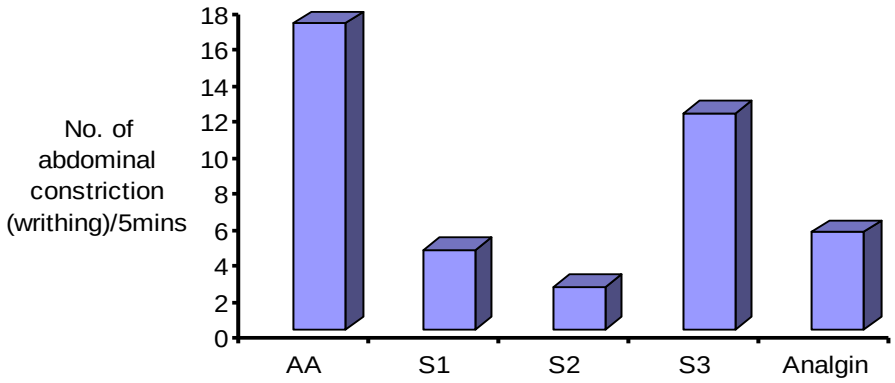


Figure 23: Analgesic activity of 50m/kg of leaves fractions of *H. acida*

Key: **AA – Acetic acid, S_1, - Sample 1, S_2 – Sample 2, S_3 – Sample 3.**

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APPENDIX A

EVALUATION OF CRUDE VEGETABLE DRUG

1. POWDERED LEAVES

a. Determination of moisture content

2g of powdered leaves of H. acida was used.

		1	2	3	
Constant weight of crucible (g)	**=**	**23.75**	**23.70**	**23.58**	
Weight of crucible and content before heating (g) (WA_1)	**=**	**25.75**	**25.70**	**25.58**	
Weight of crucible and content after heating (WA_2)	**=**	**25.28**	**25.28**	**25.27**	
Moisture content		**23.5**	**21.0**	**31.0**	

Final weight of powder after heating = wt of powder + evaporating dish before heating minus weight of powder + evaporating dish after heating

= WA1 – WA2

= 25.67 – 25-27 = 0.4g

Final weight of powder

$WA_2 – WA_1$ = Final wt of powder

= 23.67 – 25.27 = 1.59

% moisture content of powdered H. acida

$$= \frac{WA_1 - WA_2}{WA_1} \times 100\%$$

$$= \frac{2g - 1.69}{2} \times \frac{100}{1}$$

$$= \frac{0.41}{2}$$

Mean moistures content = 20.5%

b. Determination of ash value

2g of the dried powdered leaves was used (w)

		1	2	3
Constant weight of crucible (g)	**=**	**35.75**	**36.0**	**35.80**
Weight of crucible and content (g)	**=**	**37.75**	**38.00**	**37.80**

before heating (Wo)				
Weight of crucible and ash (g)	**=**	**35.84**	**35.82**	**35.82**
Weight of ash (g) w,	**=**	**03.09**	**0.18**	**0.02**

mean wt of ash = **0.096 (W_1)**

% ash value = $\frac{W_1}{W} \times \frac{100}{1}$

= $\frac{0.096}{2.00} \times 100$

= **4.8%**

c. Determination of Acid insoluble ash

		1	2	3	
Constant weight of crucible (g)	**=**	**35.70**	**35.58**	**35.81**	
Weight of filter paper W_0	**=**	**1.0**	**1.0**	**1.0**	
Weight of crucible and ash before acid treatment (g)	**=**	**35.79**	**35.76**	**35.83**	
Weight of ash (g) after acid treatment (W_1)	**=**	**1.02**	**1.05**	**1.01**	
Acid insoluble ash value	**=**	**1**	**2.5**	**0.5**	

$$\frac{W_1 - W_0}{2} \times \frac{100}{1}$$

$$\frac{\text{weight of ash}}{\text{weight of drug}} \times 100 = \frac{0.3}{2} \times \frac{100}{1}$$

Mean acid insoluble ash value = 1.5%

d. Determination of water-soluble extractive value

Weight of powdered drug (W_0) **= 5g**

Volume of 0.25% chloroform in distilled water **= 100ml**

Initial weight of beaker (W_1) **= 51.90g**

Weight of 20ml filtrate + beaker (W_2) **= 52.32g**

Weight of residue from 20ml extract W_3 **= $W_2 - W_1$**

= 52.32g – 51.90g

W_2 = 0.42g

Therefore – the weight of residue from 20ml extract (W_3) = 0.42g

If 0.42g is in 20ml extract

xg is in 100ml extract

$$xg = \frac{0.42g \times 100ml}{20ml} = 2.1g$$

Weight of residue from 100ml extract (W_4) **= 2.1g**

percentage water-soluble extractive value

$$= \frac{\text{weight of residue in 100mls}}{\text{weight of powder}} \times \frac{100}{1}$$

$$= \frac{W_4}{W_0} \times \frac{100}{1}$$

$$= \frac{2.1g}{5g} \times \frac{100}{1} = 42\%$$

% water soluble extractive value = 42%

e. Determination of alcoholic-soluble extractive value

Weight of powdered drug (W_0) **= 5g**

Volume of 90% ethanol = 100ml

Weight of evaporating dish (W_1) = 123.55g

Weight of 20ml filtrate + evaporating dish (W_2) = 123.89g

Weight of residue from 20ml extract (W_3) = $W_2 - W_1$

= 123.89 – 123.55g

W_3 = 0.34g

The weight of residue from 20ml extract (W_3) = 0.34g

If 20ml of alcoholic extract gave 0.34g of the dried residue 100mls will give xg

$$xg = \frac{100ml \times 0.34g}{20ml} = 1.7g$$

Weight of residue from 100ml extract (W_4) = 1.7g

% alcoholic extractive value

$$= \frac{\text{weight of residue in 100mls}}{\text{weight of powder}} \times \frac{100}{1}$$

$$= \frac{W_4}{W_0} \times \frac{100}{1}$$

$$= \frac{1.7g}{5g} \times \frac{100}{1} = 34\%$$

Percentage alcoholic extractive value = 34%

2. POWDERED STEM-BARK

a. Determination of moisture content

2g of powdered leaves of H. acida was used.

		1	2	3
Constant weight of crucible (g)	**=**	**20.58**	**21.0**	**20.62**
Weight of crucible and content before heating (g) (WA_1)	**=**	**22.58**	**22.40**	**22.62**
Weight of crucible and content after heating (WA_2)	**=**	**22.39**	**22.30**	**22.43**
Total loss in weight		**0.19**	**0.11**	**0.19**
Moisture content	**=**	**9.5**	**0.5**	**9.5**

Weight of moisture $= \frac{0.16}{2} \times 100$

% moisture content = 8.0%

b. Determination of ash value

		1	2	3
Constant weight of crucible	**=**	**24.46**	**24.52**	**24.49**
Weight of crucible and content before heating (g)	**=**	**26.46**	**26.38**	**26.51**
Weight of crucible and ash	**=**	**24.77**	**24.70**	**24.70**
		0.31	**0.18**	**0.21**

Mean weight of ash $= \frac{0.23}{2} \times \frac{100}{1}$

= 11.5%

c. Determination of acid insoluble ash value:

	1	2	3

Constant weight of crucible (g)	**=**	**22.30**	**22.32**	**22.40**
Weight of crucible and ash before acid treatment (g)	**=**	**24.35**	**24.35**	**24.35**
Weight of ash after acid treatment	**=**	**24.30**	**24.30**	**24.30**
Acid insoluble ash value	**=**	**0.05**	**0.05**	**0.05**

mean value = $\frac{0.05 + 0.05 + 0.05}{3}$

= **0.2**

% acid insoluble ash value = $\frac{0.15}{2}$ **x 100**

= **7.5%**

d. **<u>Determination of water-soluble extractive value:</u>**

Weight of powdered drug (W_0) = **5g**

Volume of 0.25% chloroform in distilled water = **100ml**

Initial weight of beaker (W_1) = **41.50g**

Weight of 20ml filtrate + beaker (W_2) = **41.94g**

Weight of residue from 20ml extract W_3 = **$W_2 - W_1$**

= **41.94g – 41.50g**

W_2 = **0.44g**

The weight of residue from 20ml extract (W_3) = 0.44g

If 0.44g is in 20ml extract

xg is in 100ml extract

$$xg = \frac{0.44g \times 100ml}{20ml} = 2.2g$$

Weight of residue from 100ml extract (W_4)

percentage water-soluble extractive value

$$= \frac{\text{weight of residue in 100mls}}{\text{weight of powder}} \times \frac{100}{1}$$

$$= \frac{W_4}{W_0} \times \frac{100}{1}$$

$$= \frac{2.2g}{5g} \times \frac{100}{1} = 44\%$$

% water soluble extractive value = 44%

e. Determination of alcoholic-soluble extractive value:

Weight of powdered drug (W_0) = 5g

Volume of 90% ethanol = 100ml

Weight of evaporating dish (W_1) = 123.55g

Weight of 20ml filtrate + evaporating dish (W_2) = 123.92g

Weight of residue from 20ml extract (W_3) = $W_2 - W_1$

= 123.92 – 123.55g

W_3 = 0.37g

The weight of residue from 20ml extract (W_3) = 0.37g

If 20ml of alcoholic extract gave 0.37g of the dried residue 100mls will give xg

$$\text{for } xg = \frac{100ml \times 0.37g}{20ml} = 1.85g$$

Weight of residue from 100ml extract (W_4) = 1.85g

percentage alcoholic extractive value

$$= \frac{\text{weight of residue in 100mls}}{\text{weight of powder}} \times \frac{100}{1}$$

$$= \frac{W_4}{W_0} \times \frac{100}{1}$$

$$= \frac{1.85g}{5g} \times \frac{100}{1} = 0.37\%$$

Percentage alcoholic extractive value = 0.37%

APPENDIX B

ACUTE TOXICITY STUDIES

3 animals out of 8 used for autopsy presented

1. Dose: 500mg/kg
Stock solution: 200mg/ml

Dose stock No.	Symbol	Weights	Volume of administered
1	——— •	15.0g	0.15ml (6 units)
2	• ———	10.0g	0.1ml (4 units)

2. Dose: 1,000mg/kg
Stock solution: 200mg/ml

Dose stock No.	Symbol	Weights	Volume of administered
1	• • —	21.0g	0.24ml (9 units)
2	———	15.5g	0.08ml (3 units)
3	I ———	17.5g	0.09ml (4 units)
4	——— II	20.0g	0.4ml (16 units)

3. Dose: 2,000mg/kg
Stock solution: 200mg/ml

Dose stock No.	**Symbol**	**Weights**	**Volume of administered**
1	**———I**	**23.5g**	**0.24ml (16 units)**
2	**———I**	**20.5g**	**0.4ml (16 units)**
3	**I———I**	**10.0g**	**0.2ml (8 units)**
4	**• •**	**18.5g**	**0.37ml (14 units)**

4. Dose: 3,000mg/kg

Stock solution: 200mg/ml

Dose stock No.	Symbol	Weights	Volume of administered
1	I I	17.0g	0.53ml (20 units)
2	I I I	24.0g	0.70ml (28 units)
3	I——	13.0g	0.4 (16 units)

5. Dose: 4,000mg/kg

Stock solution: 200mg/ml

Dose stock No.	Symbol	Weights	Volume of administered
1	———	26.0g	0.52ml (20 units)
2	— —	13.5g	0.27ml (10 units)
3	+	20.0g	0.4ml (16 units)

6. Dose: 5,000mg/kg

Stock solution: 200mg/ml

Dose stock No.	Symbol	Weights	Volume of administered
1	I I	25.5g	0.65mls (26 units)
2	•	25.0g	0.65mls (26 units)
3	– I	25.0g	0.65mls (26 units)

7. Dose: 10,000mg/kg

Stock solution: 200mg/ml

Dose stock No.	Symbol	Weights	Volume of administered
1	——	20.0g	1.00ml (40 units)
2	• •	26.5g	0.26ml (10 Units)
3	• ——	30.0g	0.30ml (12 units)

8. Dose: 11,000mg/kg

Stock solution: 200mg/ml

Dose stock No.	Symbol	Weights	Volume of administered
1	——I	26.0	0.87ml (34 units)
2	• •	22.0	0.81ml (32 units)
3	I I I	25.0	0.92ml (36 units)

APPENDIX C

DETAILS OF THE CALCULATION PROCEDURES OF THE STATISTICAL ANALYSES

(i) $\mathbf{X = \frac{\sum x}{N}}$ **i.e. Arithmetic mean**

Where $\mathbf{\sum x}$ **denotes the summation of the observed values**

N denotes the total number of the observations

(ii) $\mathbf{S = \pm\sqrt{\frac{\sum (x - \bar{X})^2}{n - 1}}}$ **i.e. Standard deviation**

Where $\mathbf{\bar{X}}$ **denotes the individual observations**

$\mathbf{\bar{X}}$ **denotes the arithmetic mean**

N – 1 denotes the degree of freedom

$\sqrt{\ }$ **denotes square root**

(iii) $\mathbf{S^2 = \sqrt{\frac{\sum (x - X)^2}{n - 1}}}$ **i.e. variance**

3 This is just the square of the standard derivation

(iv) $C.V = \frac{SD}{x} \times 100\%$ i.e. Co-efficient of variation

4 Where SD denotes the standard deviation

$\bar{X}$ denotes the arithmetic mean

100% denotes hundred per cent

(v) $S.E \frac{}{\sqrt{n}} = SD$ i.e. standard error

$\sqrt{\ }$

Where SD denotes the standard deviation
n denotes the square-root of the total observation

(vi) $t = \dfrac{xj - xi}{S.Ej + S.Ei}$ Dependant student's *t*–test

5 Relative to the control group

Where

j denotes the control group and

i denotes the treated group

This was compare with the tabulated student's *t*–test

Values at Df = 3; P<0.05 and P<0.01

APPENDIX D

CHRONIC TOXICITY STUDIES (STATISTICAL ANALYSIS)

Data statistical analyses for experimental feeding procedure.

a. BODY WEIGHT

Anova single factor

(Leaves pretreated)

ANOVA

Source of Variation	*SS*	*Df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	7147.263	2	3573.632	6.170162	0.011083	3.682317
Within Groups	8687.694	15	579.1796			
Total	15834.96	17				

(Leaves post-treated)

ANOVA

Source of Variation	*SS*	*Df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	7147.263	2	3573.632	6.170162	0.011083	3.682317
Within Groups	8687.694	15	579.1796			
Total	15834.96	17				

(stem-bark post-treated)

ANOVA

Source of Variation	***5.7 SS***	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	3159.806	2	1579.903	1.668622	0.221656	3.682317
Within Groups	14202.47	15	946.8311			
Total	17362.27	17				

(stem-bark post-treated)

ANOVA

Source of Variation	*SS*	*Df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	7147.263	2	3573.632	6.170162	0.011083	3.682317
Within Groups	8687.694	15	579.1796			
Total	15834.96	17				

b. <u>WATER – INTAKE</u>

(Leaves pretreated)

ANOVA

Source of Variation	***5.8 SS***	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	45.16988	2	22.58494	2.834051	0.09035	3.682317
Within Groups	119.537	15	7.969136			
Total	164.7069	17				

(Leaves post-treated)

ANOVA

Source of Variation	***5.9 SS***	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	20.66788	2	10.33394	0.90025	0.427334	3.682317
Within Groups	172.1844	15	11.47896			
Total	192.8523	17				

(stem-bark pretreated)

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	18.19201	2	9.096006	0.577682	0.573203	3.682317
Within Groups	236.1854	15	15.7457			
Total	254.3774	17				

(Stem-bark post-treated)

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	18.19201	2	9.096006	0.577682	0.573203	3.682317
Within Groups	236.1854	15	15.7457			
Total	254.3774	17				

c. FEED – INTAKE

(Leaves pretreated)

ANOVA

Source of Variation	***5.10 S S***	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	45.16988	2	22.58494	2.834051	0.09035	3.682317
Within Groups	119.537	15	7.969136			
Total	164.7069	17				

(Leaves post-treated)

ANOVA

Source of Variation	***5.11 SS***	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	20.66788	2	10.33394	0.90025	0.427334	3.682317
Within Groups	172.1844	15	11.47896			
Total	192.8523	17				

(stem-bark pretreated)

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	18.19201	2	9.096006	0.577682	0.573203	3.682317
Within Groups	236.1854	15	15.7457			
Total	254.3774	17				

(Stem-bark post-treated)

ANOVA

Source of Variation	*SS*	*Df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	18.19201	2	9.096006	0.577682	0.573203	3.682317
Within Groups	236.1854	15	15.7457			
Total	254.3774	17				

APPENDIX E

MEAN VALUE OF PRE AND POST-TREATED PER GROUPS OF RATS ON H. acida TUL. LEAVES AND STEM-BARK

Leaves

Group	**% H. acida Leaves**	**Body weight (g) (Mn ± S.E)**		**Water –intake (ml) (Mn ± S.E)**		**Feed – intake (g) (Mn ± S.E)**	
		Pre	**Post**	**Pre**	**Post**	**Pre**	**Post**
A	**25**	**219.98 ± 0.43**	**195.26 ± 0.25**	**24.22 ± 0.14**	**21.98 ± 0.29**	**22.83 ± 0.33**	**19.53 ± 0.55**
B	**50**	**191.24 ± 1.07**	**152.45 ± 0.62**	**20.22 ± 0.34**	**21.44 ± 0.3**	**16.08 ± 0.54**	**–**
C	**0**	**178.13 ± 0.136**	**199.39 ± 0.057**	**23.94 ± 0.07**	**19.18 ± 0.12**	**19.0 ± 0.451**	**19.0 ± 0.383**

Stem-bark

Group	% H. acida Leaves	Body weight (g) (Mn ± S.E)		Water –intake (ml) (Mn ± S.E)		Feed – intake (g) (Mn ± S.E)		Mn ± S.E = Mean ± Standard error n =
		Pre	Post	Pre	Post	Pre	Post	
A	25	210.24 ± 0.76	207.12 ± 0.44	24.54 ± 0.2	20.34 ± 0.34	21.09 ± 0.39	21.10 ± 0.34	
B	50	190.15 ± 0.37	158.62 ± 0.4	22.16 ± 0.13	18.78 ± 0.07	16.86 ± 0.47	–	
C	0	178.13 ± 0.136	199.39 ± 0.057	23.94 ± 0.07	19.18 ± 0.12	19.0 ± 0.451	19.0 ± 0.383	

6 i.e. number of rats per group

APPENDIX F

HAEMATOLOGY STUDIES(STATISTICAL ANALYSIS)

1. Packed cell volume (PCV) (Leaves)

ANOVA

Source of Variation	***5.12 SS***	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	381.4905	2	190.7452	6.56987	0.013263	3.982308
Within Groups	319.3667	11	29.03333			
Total	700.8571	13				

2. Packed cell volume (PCV) (Stem-bark)

ANOVA

Source of Variation	***5.13 SS***	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1.914167	2	0.9570833 3	1.671574	0.225917	3.805567
Within Groups	7.443333	13	0.5725641			
Total	9.3575	15				

3. Haemoglobin (Leaves)

ANOVA

Source of Variation	***5.14 SS***	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	44.11562	2	22.05781	7.244232	0.009835	3.982308
Within Groups	33.49367	11	3.044879			
Total	77.60929	13				

4. Hb (Stem-bark)

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	331772.4	2	165886.2	0.807852	0.466986	3.805567
Within Groups	2669449	13	205342.3			
Total	3001222	15				

5. Total Protein (TP) (Leaves)

ANOVA

Source of Variation	***5.15 SS***	*Df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	345.8027	2	172.9013	1.663241	0.230373	3.88529
Within Groups	1247.453	12	103.9544			
Total	1593.256	14				

6. TP (Stem-bark)

ANOVA

5.16 Source of Variation	*SS*	*Df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1.914167	2	0.957083	1.671574	0.225917	3.805567
Within Groups	7.443333	13	0.572564			
Total	9.3575	15				

7. Red blood cells (RBC) (Leaves)

ANOVA

5.17 Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	5.429143	2	2.714571	2.166458	0.160962	3.982308
Within Groups	13.783	11	1.253			
Total	19.21214	13				

8. Red blood cells (RBC) (Stem-bark)

ANOVA

5.18 Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	3.754167	2	1.877083	4.147663	0.040437	3.805567

Within Groups	5.883333	13	0.452564
Total	9.6375	15	

9. White blood cells (WBC) (Leaves)

ANOVA

5.19 Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	8.010143	2	4.005071	0.327487	0.727525	3.982308
Within Groups	134.527	11	12.22973			
Total	142.5371	13				

10. White blood cells (WBC) (Stem-bark)

ANOVA

5.20 Source of Variation	*SS*	*Df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	18.00417	2	9.002083	0.83108	0.457452	3.805567
Within Groups	140.8133	13	10.83179			
Total	158.8175	15				

11. Neutrophils (Neu) (Leaves)

ANOVA

5.21 Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	52.8	2	26.4	1.551282	0.254988	3.982308
Within Groups	187.2	11	17.01818			
Total	240	13				

12. Neutrophils (Neu) (Stem-bark)

ANOVA

5.22 Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	21.02083	2	10.51042	0.879155	0.438423	3.805567
Within Groups	155.4167	13	11.95513			
Total	176.4375	15				

13. Lymphocytes (Lymp) (Leaves)

ANOVA

5.23 Source of Variation	*SS*	*Df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	39.01429	2	19.50714	1.257051	0.322352	3.982308
Within Groups	170.7	11	15.51818			
Total	209.7143	13				

14. Lymphocytes (Lymp) (Stem-bark)

ANOVA

5.24 Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	30.52083	2	15.26042	1.150616	0.346665	3.805567
Within Groups	172.4167	13	13.26282			
Total	202.9375	15				

APPENDIX G

MEAN VALUE OF PRE AND POST-TREATED PER GROUPS OF RATS ON H. acida TUL. LEAVES AND STEM-BARK (BLOOD PARAMETERS)

Stem-bark

Group	% H. acida Leaves	PCV	HB	TP	RBC	WBC	NEU	LYMP
A	**25**	**50.0 ± 0.76**	**16.6 ± 0.44**	**9.17 ± 0.2**	**5.15 ± 0.34**	**12.35 ± 0.39**	**17.67 ± 0.34**	**81.2 ± 0.16**
B	**50**	**50.8 ± 0.37**	**16.9 ± 0.4**	**9.5 ± 0.13**	**6.7 ± 0.07**	**10.9 ± 0.47**	**15.3 ± 0.82**	**84.3 ± 0.43**
C	**0**	**50.6 ± 0.136**	**16.7 ± 0.057**	**9.94 ± 0.07**	**5.6 ± 0.12**	**12.36 ± 0.451**	**21.60 ± 0.383**	**76.6 ± 0.165**

Group	% H. acida Leaves	PCV	HB	TP	RBC	WBC	NEU	LYMP
A	**25**	**50.4 ± 0.43**	**16.8 ± 0.25**	**8.46 ± 0.14**	**6.28 ± 0.29**	**12.14 ± 0.33**	**14.6 ± 0.55**	**83.6 ± 0.22**
B	**50**	**38.3 ± 1.07**	**12.7 ± 0.62**	**9.1 ± 0.34**	**4.6 ± 0.3**	**12.0 ± 0.54**	**14.70 ± 0.46**	**74.7 ± 0.38**
C	**0**	**50.6 ± 0.136**	**16.7 ± 0.057**	**9.94 ± 0.07**	**5.6 ± 0.12**	**12.36 ± 0.451**	**21.60 ± 0.383**	**76.6 ± 0.165**

Leaves

Mn ± S.E = Mean ± Standard error
n = 6 i.e. number of rats per group

APPENDIX H

ANALGESIC ACTIVITY (STATISTICAL ANALYSIS)

Anova single factor

1a. Sample 1 – Acetic acid (AA)

ANOVA

Source of Variation	***5.25 SS***	*Df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	513.5417	2	256.7708	9.775539	0.018718	5.786148
Within Groups	131.3333	5	26.26667			
Total	644.875	7				

1b Sample 1 – Analgin

ANOVA

5.26 Source of Variation	*SS*	*Df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	13.33333	2	6.666667	0.153846	0.861312	5.786148
Within Groups	216.6667	5	43.33333			
Total	230	7				

2a. Sample 2 – Acetic acid

ANOVA

5.27 Source of Variation	*SS*	*Df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	782.7083	2	391.3542	62.78409	0.000287	5.786148
Within Groups	31.16667	5	6.233333			
Total	813.875	7				

2b Sample 3 – Analgin

ANOVA

5.28 Source of Variation	*SS*	*Df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	37.5	2	18.75	0.804721	0.497752	5.786148
Within Groups	116.5	5	23.3			
Total	154	7				

3a. Sample 3 – Acetic acid

ANOVA

5.29 Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	392	2	196	3.230769	0.111619	5.143249
Within Groups	364	6	60.66667			
Total	756	8				

3b. Sample 3 – Analgin

ANOVA

5.30 Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	10.88889	2	5.444444	0.0727	0.930686	5.143249
Within Groups	449.3333	6	74.88889			
Total	460.2222	8				

4. Analgin – Acetic acid

ANOVA

5.31 Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	400.1667	1	400.1667	13.04891	0.02251	7.70865
Within Groups	122.6667	4	30.66667			
Total	522.8333	5				

APPENDIX I

ANALGESIC ACTIVITY

No. of writhing / 5mins

1a. Sample 1 (50mg/kg)

No. of animals	No. of writhing
1	**16**
2	**NIL**
3	**4**
4	**NIL**
5	**3**

1b. Sample 1 (100mg/kg)

No. of animals	No. of writhing
1	**9**
2	**13**
3	**NIL**
4	**NIL**
5	**1**

2a. Sample 2 (50mg/kg)

No. of animals	No. of writhing
1	6
2	NIL
3	2
4	4
5	NIL

2b. Sample 2 (100mg/kg)

No. of animals	No. of writhing
1	5
2	NIL
3	8
4	NIL
5	NIL

3a. Sample 3 (50mg/kg)

No. of animals	No. of writhing

1	**NIL**
2	**16**
3	**1**
4	**1**
5	**17**

3b. Sample 3 (100mg/kg)

No. of animals	No. of writhing
1	**2**
2	**10**
3	**NIL**
4	**5**
5	**4**

4. Acetic acid 0.7%

No. of animals	No. of writhing
1	**28**
2	**22**
3	**9**
4	**26**

5	**NIL**

5a. Analgin (50mg/kg)

No. of animals	No. of writhing
1	**2**
2	**NIL**
3	**NIL**
4	**1**
5	**15**

5b. Analgin (100mg/kg)

No. of animals	No. of writhing
1	**NIL**
2	**NIL**
3	**1**
4	**NIL**
5	**NIL**